

THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME XXXIII

MAY-JUNE, 1957

NUMBER 3

THE GROWTH CYCLE OF VACCINIA IN HeLa CELLS CORRELATED WITH CONCURRENT CELLULAR CHANGES*

FRED W. RYDEN, Ph.D., and CHARLES C. RANDALL, M.D.

*From the Department of Microbiology, Vanderbilt University
School of Medicine, Nashville 5, Tenn.*

Numerous investigators have described the pathogenesis of vaccinia infection in experimental animals, and the nature and development of vaccinia virus have been studied extensively. Thus far, however, no definitive effort has been made to correlate replication of the virus with concurrent cytologic changes, particularly during the early stages of infection.

The advent of stable strains of mammalian cells has provided readily manipulated systems for sequential studies of host-virus relationships, and recent developments^{1,2} permit precise quantitative assay of infectivity of cytopathogenic viruses. Stable cultures of human epithelial cells (strain HeLa) have been shown to support the growth of vaccinia virus,^{3,4} and the increase of vaccinia virus in HeLa cells has been studied by means of the fluorescent antibody technique.⁵ In cultures of this nature, all cells theoretically are equally susceptible to infection. Therefore, simultaneous exposure of the cells to a sufficiently large amount of virus should result in a single sequence of infection. The problem of synchronizing viral increase in multicellular cultures has been reviewed recently by Ackermann and Francis.⁶ The usual procedure in growth cycle studies *in vitro* has been to employ a large inoculum, remove the excess virus by rinsing, and assay aliquots of the cultural fluid for viral infectivity.

During the present study, it was desired to inhibit residual extracellular virus so that the interval between infection and the formation of new virus could be determined accurately and the concentration

*Aided by a grant from the Smith, Kline & French Laboratories, Philadelphia, Pa.

This paper is a portion of the dissertation submitted by Dr. Ryden in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology, Vanderbilt University School of Medicine.

Received for publication, May 21, 1956.

of intracellular virus could be compared with the rate of release of virus from infected cells. A sequential study of vaccinia virus in the presence and absence of immune serum was undertaken, therefore, in order to achieve the desired experimental conditions. Replicate HeLa cultures were inoculated and, following a specific interval, excess virus was removed by washing. Immune serum-saline was added to one series of cultures in order to inhibit residual extracellular virus and restrict viral activity to the initially infected cells.⁷ Normal serum-saline was added to another series of cultures in order to obtain a growth curve under customary cultural conditions. At various intervals thereafter, the fluid and cellular phases of representative cultures were harvested separately and were assayed quantitatively in HeLa cultures. For histologic studies, coverglass cultures were collected at each interval and duplicate preparations were stained with hematoxylin and eosin and by Feulgen's method for desoxyribonucleic acid.

MATERIALS AND METHODS

Virus

The Western Reserve (WR) strain of vaccinia virus, obtained from the American Type Culture Collection, was employed. The virus was maintained in this laboratory by intracerebral passage in mice and was cultivated in HeLa cells.⁸ Material from the 20th passage *in vitro* was used for the present study. The infectious titer for rabbits inoculated intradermally was 10^{-5} per 0.1 ml., and the titer in HeLa cultures was 10^{-6} per 0.1 ml.

Strain of Cells

HeLa strain of cells (Gey *et al.*⁹) was employed. The cells were cultivated according to the procedures described by Syverton, Scherer, and Elwood.² The nutritive fluid has been described previously.⁸ Replicate cultures for viral studies were grown in test tubes (16 by 150 mm.) and on coverglasses (11 by 22 mm.) contained in Leighton tubes.*

Experimental Procedures

Replicate HeLa cultures were rinsed with three changes of Hanks's balanced salt solution (BSS),† and each tube received 0.1 ml. of suspension of virus containing approximately 10^5 tissue culture infectious units. The cultures were left 1 hour at room temperature, after which each culture was rinsed with five changes of wash fluid. Aliquots

* Obtained from Microbiological Associates, Bethesda, Md.

† Hanks's BSS containing 100 units of penicillin and 50 μ g. of streptomycin per ml. was employed for rinsing cultures and for dilution of virus preparations.

of the wash fluid were saved to test for viral content. One ml. of fluid medium was added to each tube and the cultures were incubated at 36° C. The fluid medium consisted of Earle-Hanks's (3:1) BSS containing either normal or immune rabbit serum in a 10 per cent concentration. The neutralizing titer of the immune serum was 1:512.⁸

Representative cultures were harvested at each designated interval following inoculation. Duplicate coverglass cultures were washed, fixed in Zenker's-acetic acid solution, and stored in 80 per cent ethanol until stained.¹⁰ Cultures for tests of infectivity were harvested in the following manner: (a) The fluid phase was transferred to a screw-capped tube, after which the cells were rinsed five times and the last wash fluid was removed; (b) 0.5 ml. of Hanks's BSS containing 10 per cent normal horse serum was added to each tube and the cells were suspended in the fluid, after which the contents of two or more tubes were pooled and transferred to a screw-capped tube. The harvested materials were stored immediately at -45° C.

For assay, harvested materials were removed from storage as required and were thawed rapidly in cold water. Tubes containing the fluid phase were centrifuged for 10 minutes at 2,000 r.p.m. in order to sediment any free cells, and the supernatant fluid was employed for assay. Each suspension of the cellular phase was transferred to a mortar and triturated for about 2 minutes. Serial 10-fold dilutions of these materials were prepared and titrations were conducted in rabbits and in HeLa cultures as described previously.⁸

Histologic Techniques

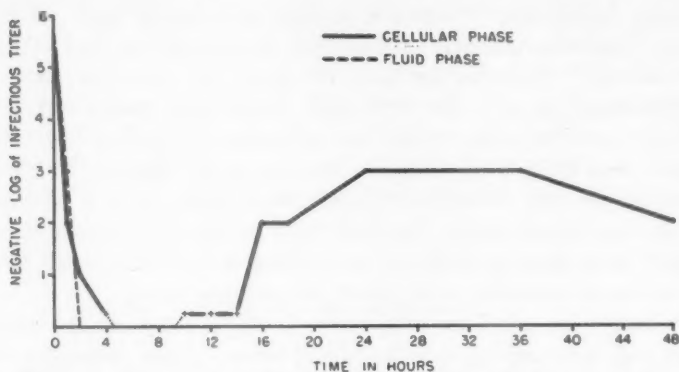
Coverglass cultures were stained with hematoxylin and eosin¹⁰ and by the method of Feulgen. Schiff's reagent was prepared according to de Tomasi,¹¹ and the Feulgen reaction was performed according to the modified method of Feulgen and Rossenbeck as detailed by Pearse.¹² Preliminary experiments showed that acid hydrolysis for 8 minutes provided optimal results. Non-hydrolyzed infected and normal cultures were included as controls.

EXPERIMENTAL RESULTS

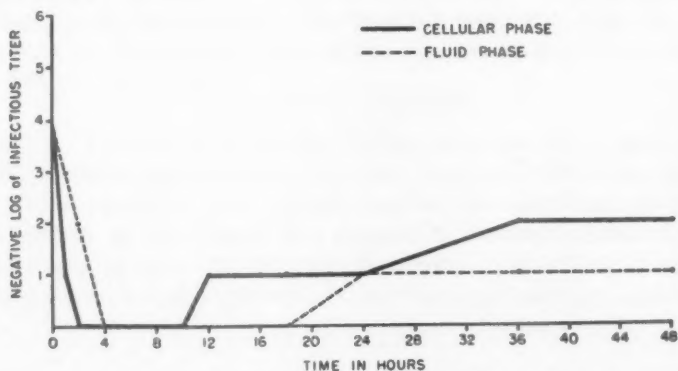
Virus inoculum that was recovered prior to the washing process titrated 10^{-3} in both rabbits and HeLa cultures. Aliquots of the wash fluid were tested in HeLa cultures; no virus was detected in the third to fifth changes of fluid, demonstrating that repeated rinsing had removed unadsorbed virus from the cultures. The results of the viral assays in rabbits and in HeLa cultures are shown in Text-figures 1 to 4.

Infectivity Tests of Cultures Containing Immune Serum
(Text-Figs. 1 and 2)

Assays of cultures containing antiserum showed that extracellular virus, both adsorbed and free, was effectively inhibited in the presence of immune serum; no virus was detected in the fluid phase by tests



Text-figure 1. Sequence of infection of vaccinia virus in HeLa cell cultures containing immune serum. Titration of fluid and cellular phases in HeLa cell cultures. Plus (+) sign indicates a reaction produced in HeLa cell cultures that were inoculated with undiluted cellular material.



Text-figure 2. Sequence of infection of vaccinia virus in HeLa cell cultures containing immune serum. Titration of fluid and cellular phases in rabbits.

in HeLa cultures, although weak dermal reactions were produced in rabbits at 24, 36, and 48 hours.

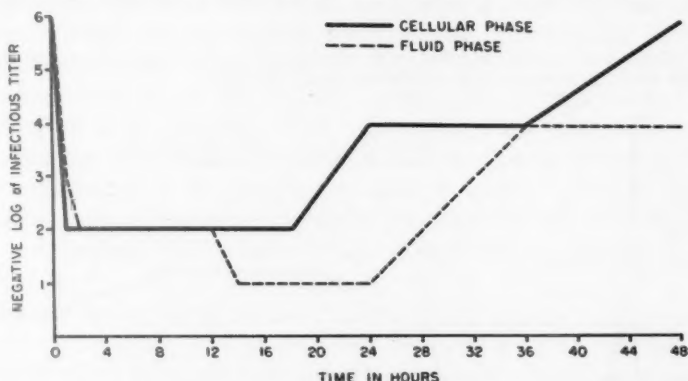
Titration in HeLa cultures of the cellular phase revealed no infective virus between 4 and 10 hours after inoculation. At 10, 12, and 14 hours virus was present in small quantities in undiluted cellular

suspensions. During the next 2 hours the virus concentration increased abruptly to a titer of 10^{-2} and then increased more slowly between 18 and 24 hours to a maximal titer of 10^{-3} . This level was maintained until the 36-hour interval, after which the titer slowly decreased.

Titration in rabbits of the cellular phase revealed no newly formed virus until an interval of 12 hours, 2 hours later than virus was detected in HeLa cultures. Moderately severe dermal reactions were produced in a dilution of 10^{-1} until an interval of 36 hours, when a 10-fold increase in viral concentration occurred. This level was maintained through the 48-hour interval, although the dermal reactions decreased in severity.

Infectivity Tests of Cultures Containing Normal Serum
(Text-Figs. 3 and 4)

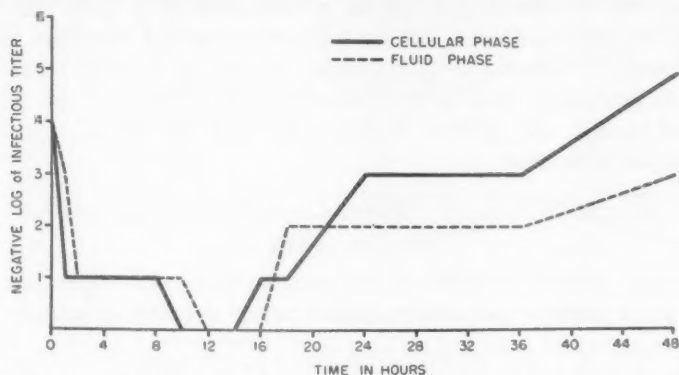
Assays in HeLa cultures of the cellular phase showed a titer of 10^{-2} for a period of 18 hours; plaque counts revealed a decrease in



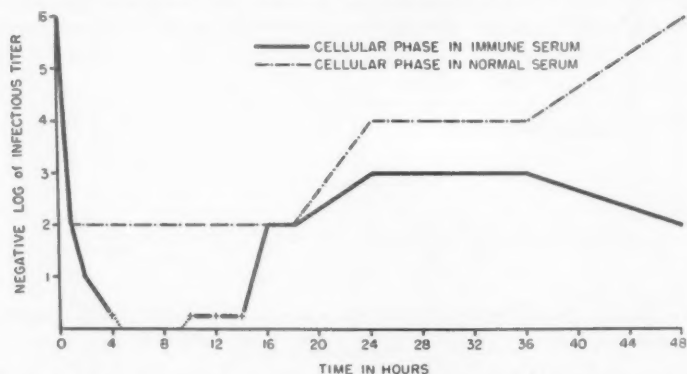
Text-figure 3. Sequence of infection of vaccinia virus in HeLa cell cultures containing normal serum. Titration of fluid and cellular phases in HeLa cell cultures.

viral concentration between log 1 and log 2 during the initial period of 10 hours (corresponding to the "lag phase" which was demonstrated in the presence of immune serum), but this variation in activity was not shown in the graph. Between 18 and 24 hours the titer increased to 10^{-4} and then remained constant until the 36-hour interval, after which the titer rose to a maximum of 10^{-6} . Virus in the fluid phase titered 10^{-2} for 10 hours, decreased temporarily in concentration, and then increased 1,000-fold between 24 and 48 hours after inoculation. A maximal titer of 10^{-5} was attained between 48 and 72 hours.

Titration in rabbits produced approximately the same results as in HeLa cultures, although the viral titers ranged one 10-fold lower, and a drop in viral concentration occurred between 8 and 14 hours, resulting in a graphic profile not unlike that which was obtained in the presence of immune serum (Text-figs. 1 and 4).



Text-figure 4. Sequence of infection of vaccinia virus in HeLa cell cultures containing normal serum. Titration of fluid and cellular phases in rabbits.



Text-figure 5. Sequence of infection of vaccinia virus in HeLa cell cultures in the presence and absence of immune serum. Titration of the cellular phases in HeLa cell cultures. Plus (+) sign indicates a reaction produced in HeLa cell cultures that were inoculated with undiluted cellular material.

The experimental results show that vaccinia infection in HeLa cells consisted of two distinct phases: (a) an inceptive period characterized by the disappearance of the infectious parent virus after entry into the cell, and (b) a period of viral increase characterized by the appearance of progeny and subsequent step-wise increase in

viral concentration. A constant level of virus was present between 24 and 36 hours and another period of viral increase ensued in the presence of normal serum to produce a maximal titer at 48 hours after inoculation. Viral reproduction ceased in the presence of immune serum, and the concentration of virus decreased between 36 and 48 hours. The sequence of infection in the presence and absence of immune serum is shown clearly in Text-figure 5, which is comprised of portions of Text-figures 1 and 2.

Morphologic Changes in Infected Cells

Elementary bodies were not distinguished with certainty during the course of infection, either in cultures stained with hematoxylin and eosin or by Feulgen's method. Rare intracytoplasmic inclusion bodies were noted first at 6 hours after inoculation. They generally occurred singly, lay close to the nuclear membrane, varied from round to ovoid, and usually were surrounded by a faint halo. Figure 2 shows Feulgen-stained cells containing well defined perinuclear inclusions which were Feulgen-positive and appeared reddish lavender. Examination of the nuclei suggests that a change of some kind occurred in the nuclear membrane adjacent to the inclusion bodies. The nucleus marked A shows a definite concavity which approximates the position of the inclusion, and the nuclear membrane is stained densely in this region. The membrane of the nucleus marked B appears irregular and shrunken near the site of the inclusion. Definite inclusion bodies also were present at 6 hours in cultures stained with hematoxylin and eosin (Fig. 3).

Viral inclusions occurred more frequently as infection progressed, but further cellular changes were not observed until an interval of 18 hours when scattered cells showed cytoplasmic retraction accompanied by separation of adjacent cells. At 24 hours numerous small foci of degeneration were evident which rapidly enlarged peripherally, with coalescence of neighboring plaques (Figs. 4 to 6).

Essentially similar cytopathogenic changes were noted after an interval of 36 hours in cultures with and without immune serum, and within 48 hours after inoculation the cultures containing normal serum consisted almost entirely of injured and necrotic cells (Fig. 7). In cultures containing immune serum, however, degeneration of cells ceased after approximately 36 hours, and at 48 hours (Fig. 8) the cultures appeared relatively healthy in areas surrounding the original degenerative foci. The amelioration of damage to cells in the presence

of immune serum coincided with apparent cessation of viral multiplication after an approximate interval of 36 hours.

In summary, viral inclusion bodies which stained Feulgen-positive were present 6 hours after inoculation, preceding the appearance of infectious virus by about 4 hours. Early cytopathogenic changes occurred simultaneously with the first significant rise in concentration of newly formed virus between 18 and 24 hours, and viral increase was associated with progressive cellular degeneration. Marked destruction of cultures containing normal serum occurred within 48 hours after inoculation, whereas viral multiplication and cellular damage ceased simultaneously at about 36 hours in cultures containing immune serum.

DISCUSSION

The experimental data show that vaccinia virus underwent a single sequence of infection in cultures containing immune serum. Virus that entered the cells during the first hour of contact theoretically was protected from the action of the antiserum, whereas extracellular virus was effectively neutralized, and viral activity was limited thereby to the initially infected cells. The use of immune serum eliminated the masking effect of residual viral inoculum and allowed the detection of the earliest viral progeny. Moreover, disruption of the cells by freezing and thawing and by trituration prior to assay permitted detection of newly formed intracellular virus. That disruption of infected cells is important in studies of this nature has been shown with the virus of Western equine encephalomyelitis, which appears extracellularly about 3 hours after infection of fibroblasts of chick embryo,¹³ whereas Rubin *et al.*¹⁴ disrupted similarly infected cells by means of ultrasonic vibration and demonstrated new virus within 1 to 2 hours after inoculation.

Although the early stages of viral reproduction were shown to best advantage in cultures containing immune serum, the pattern of growth was similar in cultures with and without immune serum throughout the 36-hour interval, when the viral concentration was indicated by the data to be the cumulative effect of three infectious cycles. The decrease in the level of virus in the cells after this period in the presence of immune serum suggests at least two possibilities: (a) The infected cells could no longer support viral growth due to the injurious action of the virus, and transmission of infection via the fluid medium to any remaining healthy cells was inhibited by the antiserum; (b) immune antibodies entered the cells either before or subsequent to the onset of cytopathologic changes and eventually suppressed

viral reproduction. Perhaps both phenomena functioned concurrently.

Titration *in vitro* and *in vivo* produced similar growth curves, but HeLa cultures proved superior to rabbits in many respects. Variation among the animals to infection was obvious, whereas accurate end points of titration could be readily determined and reproduced *in vitro*. The rabbits were less sensitive in general to small amounts of virus than were the HeLa cultures, although rabbit skin appeared more susceptible to infection with virus that had been exposed to immune serum. Virus was not detected in the fluid phase of cultures containing immune serum by assay in HeLa cultures, but small amounts of virus were demonstrated at 24, 36, and 48 hours *in vivo*. It appears that antiserum which remained associated with the virus masked its infectivity but that this effect became diminished more readily *in vivo* than *in vitro*. On the other hand, HeLa cultures were shown to be more sensitive than rabbit skin to infection with material from the cellular phase of cultures containing immune serum. The situation is not readily explained and requires further investigation. Sabin¹⁵ has shown that infective vaccinia virus can be recovered from inactive antiserum-virus mixtures by means of ultracentrifugation. It would be of interest to subject the preparations of the cellular phase to this process prior to tests for infectivity.

Comparison of the growth curve of vaccinia virus in HeLa cells with the findings of other workers reveals certain similarities and differences. Briody and Stannard¹⁶ concluded that vaccinia virus in the chorioallantois of chick embryos had a "one-step growth curve" of 8 hours and multiplied in a step-wise fashion; a slight increase in viral titer was observed about 8 hours after inoculation and another usually more marked increase occurred at 17 hours, which was followed by a gradual increase to a maximal titer at 43 hours. Anderson¹⁷ reported that vaccinia virus in chick chorioallantoic membrane increased at a logarithmic rate between 10 and 24 hours after inoculation; a modified tissue culture method also was employed which the author believed demonstrated an initial "eclipse period" of 8 hours. Crawford and Sanders¹⁸ observed rapid increase of vaccinia virus in flask cultures of rabbit skin between 10 and 40 hours after inoculation. Apparently none of the above authors removed excess virus following inoculation, and the pattern of viral growth during the later stages of infection indicates that overlapping cycles of infection occurred.

Accurate determination of intracellular reproduction requires either that the threshold level of extracellular virus be known or that virus

which does not enter the host cell be removed. Assays *in vitro* of cells and fluid from cultures containing normal serum showed a titer of 10^{-2} between 2 and 12 hours after inoculation; during the same interval, extracellular virus in the presence of immune serum was inhibited and virus that entered the cells became inapparent. The findings demonstrate that a portion of the inoculum became fixed firmly enough to the cells to resist removal during a total of ten changes of wash fluid (including the rinsing process when the cultures were harvested). Another feature concerning cell-virus interaction during the early hours of contact is shown by comparison of the fluid and cellular levels of virus in cultures containing normal serum. A certain amount of adsorbed virus failed to penetrate the cells and was not removed by washing but gradually eluted from the cellular surfaces, resulting in an equilibrium between free and adsorbed virus. Ackermann *et al.*¹⁹ recently described two forms of influenza virus during infection of sections of chick chorioallantois. One form could be removed from tissues and did not function in viral multiplication; another form initiated infection and was not recoverable during the latent period. The bound infectious virus (BIV) and the initiating activity (IA) virus of Ackermann and coworkers probably represents the same phenomenon, with different nomenclature, that was observed with vaccinia virus in HeLa cultures during the present study.

Concerning the release of newly formed virus from the cells, harvesting and titrating the cellular and fluid phases separately disclosed that the viral content of the fluid phase in cultures containing normal serum lagged behind the concentration of the cellular phase. The results show that infectious progeny of the virus did not emerge from the cells at the same rate that it was formed and demonstrate that in order to obtain a precise pattern of viral development in tissue cultures both the fluid and cellular phases must be assayed.

The occurrence of intracytoplasmic inclusion bodies prior to detection of infectious progeny of the virus suggests that either the virus was immature when first visualized or that inclusion body formation preceded demonstrable viral replication. Although the vaccinal inclusions consist of particulate virus in tissues examined during the late stages of infection,^{20,21} the present observations do not warrant the assumption that the earliest inclusions contain elementary bodies. Rather, the morphologic changes that were noted in Feulgen-stained HeLa cells present the possibility that vaccinal infection induces alteration of the nuclear membrane in some manner with subsequent release of nucleoplasm for assimilation by replicating cytoplasmic virus. Recent findings have supported the conclusions of earlier

workers that vaccinia virus multiplies exclusively in the cytoplasm. Gaylord *et al.*²¹ observed no intranuclear elementary bodies in electron photomicrographs of chorioallantoic cells infected by vaccinia virus, and Noyes and Watson,⁵ using the fluorescent antibody technique, found no particulate antigen in the nuclei of infected HeLa cells. Although it has been known for some time that vaccinia virus is composed in part of desoxyribonucleic acid,²² the means by which this substance is obtained and incorporated by the virus remains obscure. The present findings hint that viral activity may influence the release of nuclear material into the cytoplasm, providing a pool of desoxyribonucleic acid, available for utilization by the virus. Bland and Robinow,²³ however, considered and discarded the possibility that nuclear budding plays a rôle in the formation of vaccinia inclusions. During the present study, nuclear budding was observed in both infected and uninfected HeLa cells; nuclear fragments in the cytoplasm stained heavily in Feulgen preparations and did not resemble the viral inclusions (Fig. 3). Furthermore, there is concrete evidence that nuclei of mammalian cells continuously expel nuclear material into the cytoplasm. Walker and Tozer²⁴ first observed nuclear extrusion in planarian cells and in rabbit leukocytes. More recently, Duryee and Doherty²⁵ showed that nuclei of amphibian oocytes evert their contents into the cytoplasm, and found that the inclusions of the renal adenocarcinoma of the frog arise directly from the nucleoplasm. Finally, Pomerat *et al.*²⁶ have demonstrated by time lapse cinematography that tissue culture cells continuously extrude nuclear blebs into the cytoplasm. Although viral infection might accelerate such nuclear extrusions, the essential problem is not so much how nuclear components are contributed to the cytoplasm as the manner in which they are assimilated by replicating cytoplasmic viruses.

Comparison of inclusion body formation in HeLa cells with the comprehensive study by Bland and Robinow²³ of vaccinia reproduction in cultures of rabbit corneal cells is rather difficult due to differences in cell types, methods of fixation, and staining techniques. These authors fixed plasma clot cultures in osmic acid and stained by Giemsa's method. Photographs of infected cells showed little nuclear detail and the viral material stained densely. Inclusion bodies of five successive types were described: small and large homogeneous bodies and small, medium, and large "networks." Feulgen-positive inclusions were noted several hours after infection. The earliest inclusion bodies that were noted in HeLa cells during the present study corresponded in appearance to the large homogeneous bodies and

small networks described by Bland and Robinow, but they stained more lightly and showed more distinct internal structure (Fig. 3). Apparent differences between the results of these investigations indicate the need for further study concerning the tinctorial properties of vaccinia virus during reproduction.

The degenerative changes in HeLa cells corresponded more closely to viral multiplication than did the number and appearance of inclusion bodies. Increased cytoplasmic retraction and vacuolation coincided with the first significant rise in viral concentration, and almost complete disintegration of the cultures containing normal serum occurred coincidentally with maximal production of virus. In the presence of immune serum, the degenerative process did not continue after detectable viral reproduction ceased, suggesting that active viral replication and not merely the presence of preformed virus was necessary for continued degeneration. It seems plausible that entry of immune serum into the cells inhibited the virus and secondarily protected against further cellular injury.

SUMMARY

The growth cycle of vaccinia virus in HeLa cells in the presence and absence of immune serum has been investigated. The composite results are as follows: (a) Viral reproduction was characterized by a latent period of 8 to 10 hours followed by successive increases of virus to produce a maximal titer at 48 hours after inoculation; (b) infective virus was not detected in the cells from the fourth to the tenth hour after inoculation, although Feulgen-positive inclusion bodies were present at 6 hours; (c) the release of progeny of the virus from the cells lagged several hours behind the formation of infective intracellular virus, and marked increase in extracellular virus occurred coincidentally with progressive cytopathologic changes, resulting in destruction of the cultures within 48 hours after inoculation; (d) similar growth curves were obtained by quantitative assays in rabbits and in HeLa cultures, but the latter method proved superior in many respects.

REFERENCES

1. Dulbecco, R. Production of plaques in monolayer tissue cultures by single particles of an animal virus. *Proc. Nat. Acad. Sc.*, 1952, **38**, 747-752.
2. Syverton, J. T.; Scherer, W. F., and Elwood, P. M. Studies on the propagation *in vitro* of poliomyelitis viruses. V. The application of strain HeLa human epithelial cells for isolation and typing. *J. Lab. & Clin. Med.*, 1954, **43**, 286-302.
3. Scherer, W. F., and Syverton, J. T. The viral range *in vitro* of a malignant human epithelial cell (strain HeLa, Gey). I. Multiplication of herpes simplex, pseudorabies, and vaccinia viruses. *Am. J. Path.*, 1954, **30**, 1057-1073.

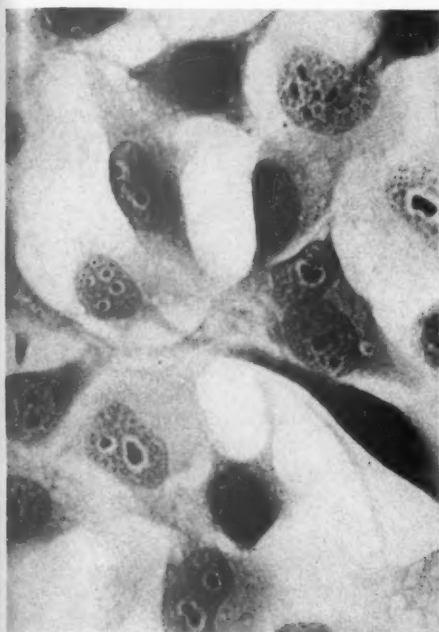
4. Tyrrell, D. A. J. New tissue culture systems for influenza, Newcastle disease and vaccinia viruses. *J. Immunol.*, 1955, **74**, 293-305.
5. Noyes, W. F., and Watson, B. K. Studies on the increase of vaccinia virus in cultured human cells by means of the fluorescent antibody technique. *J. Exper. Med.*, 1955, **102**, 237-242.
6. Ackermann, W. W., and Francis, T., Jr. Characteristics of viral development in isolated animal tissues. *Advances in Virus Research*, 1954, **2**, 81-108.
7. Ellis, E. L., and Delbrück, M. The growth of bacteriophage. *J. Gen. Physiol.*, 1939, **22**, 365-384.
8. Ryden, F. W., and Randall, C. C. A study of three strains of vaccinia virus in stable cell strains L, LLC-M₁ and HeLa. *Am. J. Path.*, 1957, **33**, 293-311.
9. Gey, G. O.; Coffman, W. D., and Kubicek, M. T. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Research*, 1952, **12**, 264-265.
10. An Introduction to Cell and Tissue Culture. Burgess Publishing Co., Minneapolis, 1955, p. 120.
11. de Tomasi, J. A. Improving the technic of the Feulgen stain. *Stain Technol.*, 1936, **11**, 137-144.
12. Pearse, A. G. E. Histochemistry: Theoretical and Applied. Little, Brown & Co., Boston, 1953, p. 425.
13. Dulbecco, R., and Vogt, M. One-step growth curve of Western equine encephalomyelitis virus on chicken embryo cells grown *in vitro* and analysis of virus yields from single cells. *J. Exper. Med.*, 1954, **99**, 183-199.
14. Rubin, H.; Baluda, M., and Hotchin, J. E. The maturation of Western equine encephalomyelitis virus and its release from chick embryo cells in suspension. *J. Exper. Med.*, 1955, **101**, 205-212.
15. Sabin, A. B. The mechanism of immunity to filterable viruses. I. Does the virus combine with the protective substance in immune serum in the absence of tissue? *Brit. J. Exper. Path.*, 1935, **16**, 70-84.
16. Briody, B. A., and Stannard, C. Studies on vaccinia virus. I. The development of hemagglutinating and infective particles in the chorioallantois of the chick embryo. *J. Immunol.*, 1951, **67**, 403-411.
17. Anderson, S. G. The growth curve of vaccinia virus on the chorioallantois. *Australian J. Exper. Biol. & M. Sc.*, 1954, **32**, 633-639.
18. Crawford, G. N. C., and Sanders, F. K. The multiplication of vaccinia virus in tissue cultures of adult rabbit skin. *Quart. J. Micr. Sc.*, 1952, **93**, 119-132.
19. Ackermann, W. W.; Ishida, N., and Maassab, H. F. Growth characteristics of influenza virus concerning the binding of virus by host cells. *J. Exper. Med.*, 1955, **102**, 545-554.
20. Goodpasture, E. W.; Woodruff, A. M., and Buddingh, G. J. Vaccinal infection of the chorio-allantoic membrane of the chick embryo. *Am. J. Path.*, 1932, **8**, 271-281.
21. Gaylord, W. H., Jr.; Melnick, J. L., and Bunting, H. Intracellular development of vaccinia virus. *Proc. Soc. Exper. Biol. & Med.*, 1952, **80**, 24-27.
22. Hoagland, C. L.; Lavin, G. I.; Smadel, J. E., and Rivers, T. M. Constituents of elementary bodies of vaccinia. II. Properties of nucleic acid obtained from vaccinia virus. *J. Exper. Med.*, 1940, **72**, 139-147.
23. Bland, J. O. W., and Robinow, C. F. The inclusion bodies of vaccinia and their relationship to the elementary bodies studied in cultures of the rabbit's cornea. *J. Path. & Bact.*, 1939, **48**, 381-403.

24. Walker, C. E., and Tozer, F. M. Observations on the history and possible function of the nucleoli in the vegetative cells of various animals and plants. *Quart. J. Exper. Physiol.*, 1909, 2, 187-200.
 25. Duryee, W. R., and Doherty, J. K. Nuclear and cytoplasmic organoids in the living cell. *Ann. New York Acad. Sc.*, 1954, 58, 1210-1231.
 26. Pomerat, C. M.; Lefeber, C. G., and Smith, McD. Quantitative cine analysis of cell organoid activity. *Ann. New York Acad. Sc.*, 1954, 58, 1311-1321.
 27. Black, F. L., and Melnick, J. L. Micro-epidemiology of poliomyelitis and herpes-B infections: spread of the virus within tissue cultures. *J. Immunol.*, 1955, 74, 236-242.
-

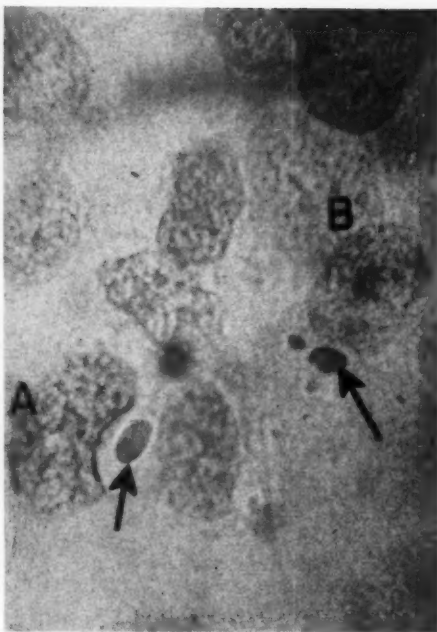
LEGENDS FOR FIGURES

- FIG. 1. Uninfected HeLa cells. Hematoxylin and eosin stain. $\times 598$.
- FIG. 2. Feulgen-stained cells, 6 hours after inoculation. Typical inclusions and indentation of the adjacent nuclear membranes may be noted. $\times 1,134$ (oil immersion).
- FIG. 3. Six hours. A discrete inclusion is shown. The central portion is opaque and the margin is dense. This may be compared with the spherical cytoplasmic bodies in the neighboring cell; these bodies possess dark centers resembling nuclear chromatoid material and are considered to be nuclear fragments. Hematoxylin and eosin stain. $\times 598$.
- FIG. 4. Twenty-four hours. Between 18 and 24 hours after inoculation numerous foci of degeneration became apparent. Hematoxylin and eosin stain. $\times 252$.

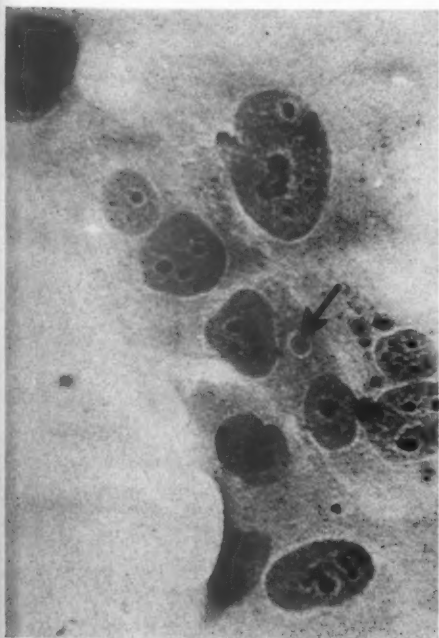




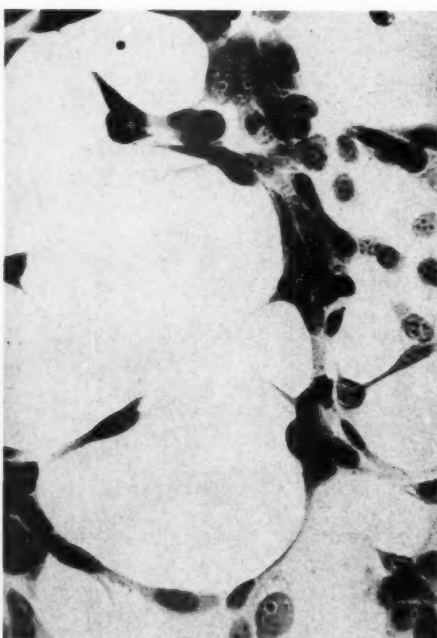
1



2



3



4

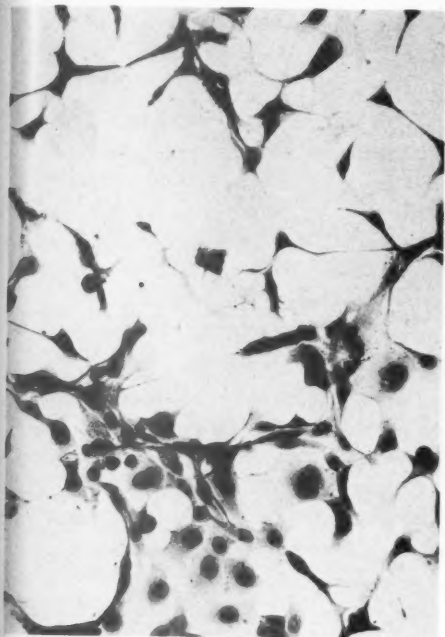
FIG. 5. Twenty-four hours. The progressive involvement of the cultures in the presence of normal serum as infection progressed is depicted in Figures 4 to 6. Black and Melnick²⁷ coined the term micro-epidemiology to designate the spread of viral infection in tissue cultures. Hematoxylin and eosin stain. $\times 252$.

FIG. 6. Thirty-six hours. Hematoxylin and eosin stain. $\times 126$.

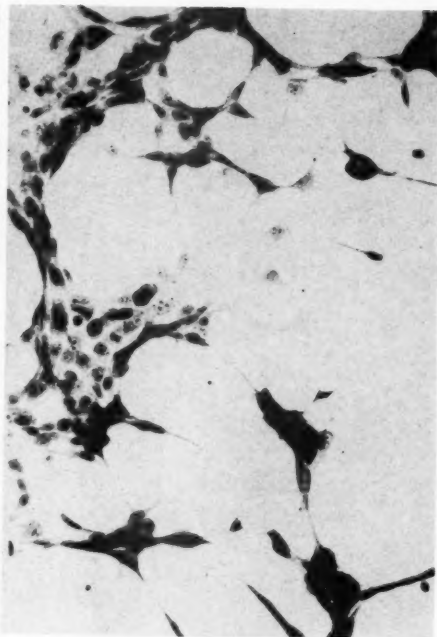
FIG. 7. Forty-eight hours. Infected cultures in the presence of normal serum underwent almost complete disintegration within 48 hours after inoculation. The remaining cells were either badly damaged or necrotic. Hematoxylin and eosin stain. $\times 252$.

FIG. 8. Forty-eight hours. Cultures in the presence of immune serum showed no further cellular damage between 36 and 48 hours, and some areas of the cultures appeared relatively healthy in contradistinction to the marked destruction which occurred in cultures containing normal serum (Fig. 7). Hematoxylin and eosin stain. $\times 252$.

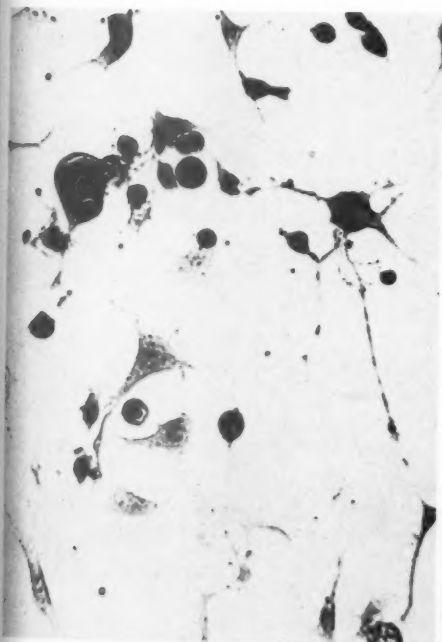




5



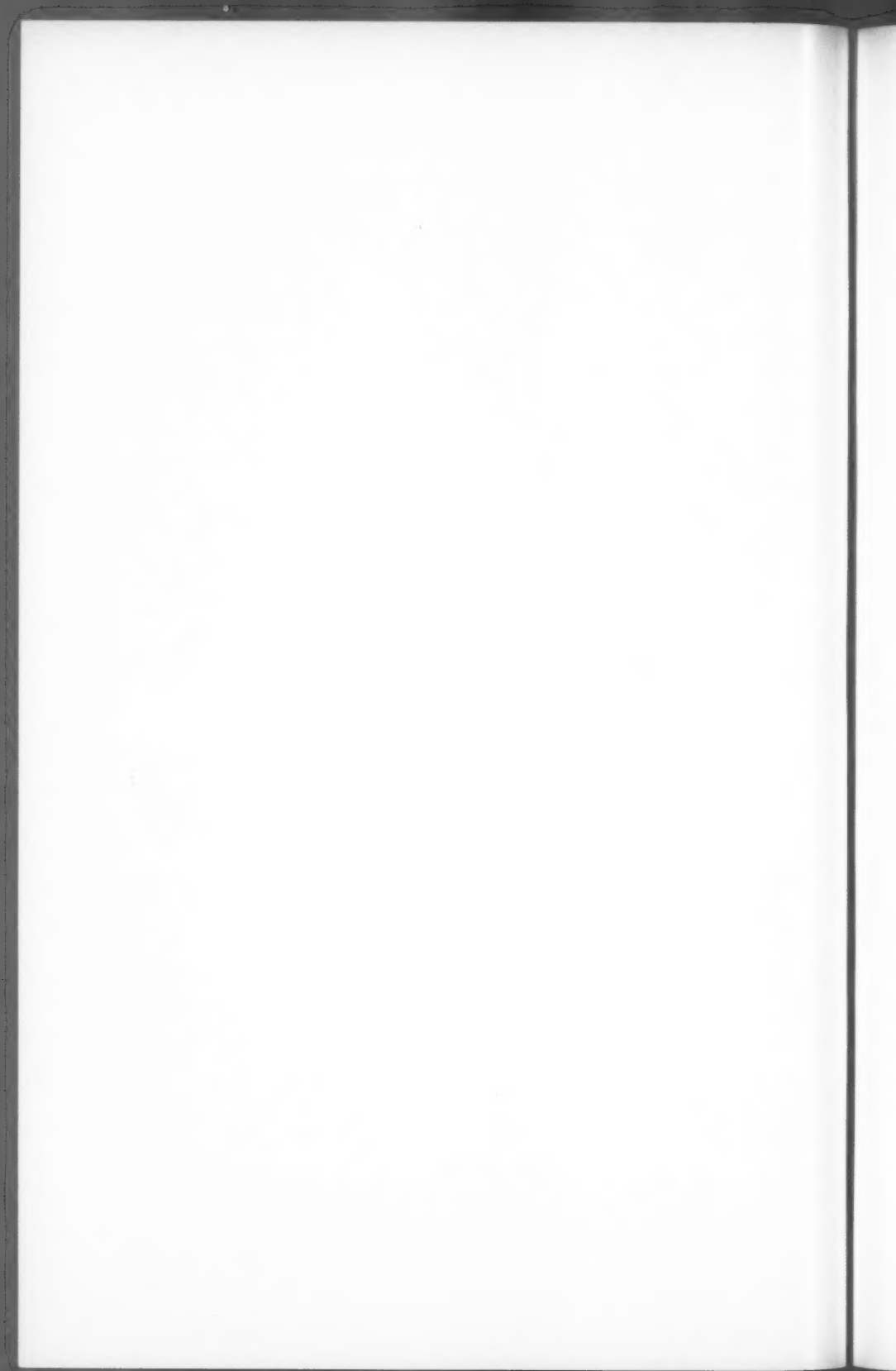
6



7



8



EXPERIMENTAL CRYPTOCOCCOSIS (TORULOSIS)*

SEYMOUR LEVINE, M.D.†; H. M. ZIMMERMAN, M.D., and ANTONIO SCORZA

From the Laboratory Division, Montefiore Hospital, New York 67, N.Y.

Cryptococcosis (torulosis), caused by the pathogenic yeast-like fungus *Cryptococcus neoformans*, is a disease which presents numerous unsolved problems relative to portal of entry, immunology, histopathology, selectivity of localization, natural history, diagnosis, and therapy. Studies of human material¹⁻³ have indicated the respiratory tract as the portal of entry, and have shown poor immunologic responses to the infection. The histopathology is characterized by a granulomatous inflammation or by a minimal inflammatory reaction, with preferential localization in the central nervous system. The disease tends to progress, although instances of healing are recorded. The study of human material has obvious limitations for solution of the stated problems, and the experimental investigations recorded here were designed to shed additional light on some of them, particularly the histopathologic features of early lesions and the natural history of infections produced by different routes of inoculation.

METHODS

Three pathogenic strains of *C. neoformans* were supplied by Dr. Rhoda Benham. Strain 1499.20 and strain 1499.57 were isolated from human cases of cryptococcosis in 1951 and 1952, respectively, and strain 1499.62 (Sanfelice's strain) was received from the Central-bureau voor Schimmelcultures in 1953. Cultures were grown in Mycophil broth‡ at room temperature for 3 to 5 days, including 16 hours of mechanical agitation. The broth cultures were centrifuged and the cells were suspended in saline solution. Samples of the saline suspension plated immediately prior to inoculation on Sabouraud's agar for incubation at room temperature and on blood agar for incubation at 37° C. demonstrated no fungal or bacterial contaminants. When dilutions of the inoculum were required, sterile saline solution was employed as diluent. The yeast cells were counted with the aid of a Levy chamber.

Subcutaneous inoculations were made at the midline of the lower

* This investigation was carried out while Dr. Levine held a Pathology Traineeship award from the National Institute of Neurological Diseases and Blindness, U.S. Public Health Service.

Received for publication, June 18, 1956.

† Now at St. Francis Hospital, Jersey City 2, N.J.

‡ Obtained from the Baltimore Biological Laboratory, Baltimore, Md.

abdomen, 1 day after shaving. Intraperitoneal inoculations were made into the lower abdomen, without prior shaving. Intracerebral inoculations were made with a 25 gauge hypodermic needle. The skull was entered just behind and medial to the ear, with the needle directed anteriorly, ventrally, and toward the midline. No anesthetic was required. The volume of each inoculation was 0.1 cc.

Necropsies were done on all animals that died during the experiment or that were sacrificed by ether inhalation. Cultures were prepared on Sabouraud's agar and blood agar, from brain, lung, liver, and any subcutaneous lesions. Tissues were fixed in 10 per cent formalin and embedded in paraffin. Routine sections of brain, lung, heart, liver, spleen, kidney, and skin were stained with hematoxylin and eosin and with the periodic acid-Schiff (PAS) stain.

RESULTS

Subcutaneous Inoculation

Twenty-nine female A albino mice (average age, 3½ months) were inoculated subcutaneously with 13 million yeast cells in a saline suspension from a 3-day broth culture of *C. neoformans*, strain 1499.20. They were sacrificed at intervals of 1 day to 5 months.

On the first day after inoculation, palpable nodules were present at the site of injection in all animals. Microscopically, there were collections of closely packed polymorphonuclear neutrophils in the subcutaneous adipose tissue. Yeast cells were visible in sections stained with hematoxylin and eosin, but were rendered more prominent by the PAS stain. They were round or oval with occasional buds. The cell wall was deeply stained by the Schiff reagent and frequently showed a deep indentation or fold. Rarely was the presence of soluble capsular material indicated by an unstained space or halo. The mass of inflammatory cells was traversed by persisting strands of connective tissue and cutaneous maximus muscle. A few polymorphonuclear leukocytes were present in the surrounding adipose tissue and the overlying dermis, but not in the subjacent muscle of the abdominal wall.

On the second day, the inflammatory subcutaneous mass was larger, well demarcated, and composed of innumerable polymorphonuclear leukocytes and yeast cells with complete dissolution of tissue, that is, a true abscess (Fig. 1). The adipose tissue around the abscess was edematous, and contained numerous spindle-shaped fibroblasts. There were diffuse infiltrations of lymphocytes and polymorphonuclear leukocytes, and small collections of these cells surrounded congested

capillaries. In some animals, other smaller subcutaneous abscesses were present. Each of the abscesses contained many yeast cells, and yeasts were not seen outside of the abscesses. Probably, movement of the needle during injection of the suspension was responsible for the multiple abscesses.

On the third day, the subcutaneous abscesses reached maximal size (0.5 to 0.7 cm. in diameter). There was a tendency for the yeast cells to be clustered in the center of the abscess. The overlying dermis and epidermis were thin and necrotic (Fig. 2).

By the fifth day, one third of the animals showed ulcerated lesions and the necrotic skin was separated or desquamated. Expelled with it was part of the contents of the abscess, namely, necrotic polymorphonuclear leukocytes and large numbers of yeast cells. The base of the ulcer was usually well demarcated by a wall of inflammatory cells and young fibrous tissue. The epidermis at the margins of the lesion showed downward proliferation. By the seventh day, ulceration was detected in one half of the animals (Fig. 3).

On the 13th and 20th days, proliferation of capillaries and fibroblasts and infiltration by lymphocytes and plasma cells were much more conspicuous around the abscesses. An occasional multinucleated giant cell of Langhans' type was observed. The yeast cells were concentrated in the center of the abscesses which now contained many macrophages with vacuolated cytoplasm (Fig. 4). Some of the macrophages contained yeast cells. The striated muscle of the abdominal wall showed little or no alteration despite the intense inflammation in the subcutis immediately above.

After the 13th day, more and more of the palpable nodules disappeared. Sections of skin after 20 days showed nothing beyond fibrosis with a few inflammatory cells; no yeast cells were demonstrated (Fig. 5).

Only two of the 29 mice showed signs of systemic illness during the experiment. At the end of 1 month they were sacrificed. Disseminated cryptococcosis was confirmed by cultures and histologic sections. Extensive involvement of the brain was demonstrated in both animals, and involvement of heart, liver, and kidney in one. Sections of the abdominal wall of one of these mice showed a healing cryptococcal granuloma involving peritoneum and abdominal muscles. It is likely that dissemination in this case was due to an inadvertent peritoneal and intramuscular inoculation.

Absence of disseminated infection in the other 27 mice was indicated by histologic examinations of brain, heart, lung, liver, spleen, and kidney. In addition, cultures of brain, lung, and liver of 15 mice

were sterile. The necropsies were performed and the cultures made on the last eight mice after an interval of 5 months.

Pure cultures of *C. neoformans* were recovered from the subcutaneous abscesses of two animals during this experiment. Occasionally bacteria were cultured from the ulcerated lesions. (Including all the experiments in which subcutaneous abscesses were produced, pure cultures of *C. neoformans* were recovered on 15 occasions.)

In contrast with the results just described, subcutaneous inoculation of heat-killed cryptococcal cells elicited a transitory polymorphonuclear infiltrate on the first day. The infiltrate and the killed yeast cells could not be detected in sections by the third day.

Intracerebral Inoculation

Seventeen female mice of the same strain and average age were inoculated intracerebrally with the same dosage of cryptococci in saline suspension as was used for the subcutaneous inoculations. Within 5 days, all of these animals died or were sacrificed when moribund. In order to increase the survival periods, groups of five mice were inoculated with four serial 10-fold dilutions of the yeast cell suspension.

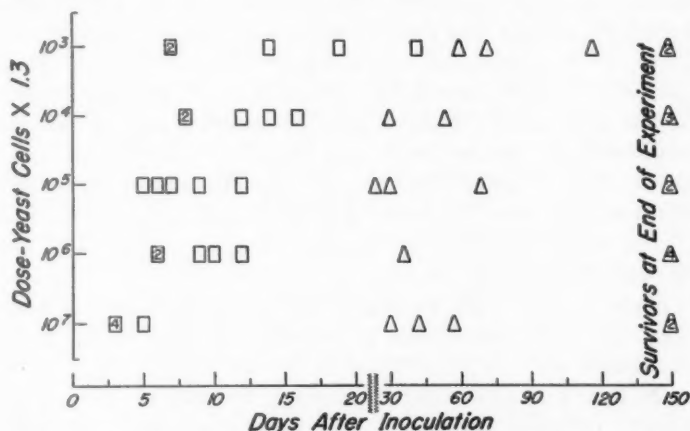
On the first day after inoculation, sections revealed small, irregular, cystic spaces in the cerebral parenchyma. The spaces contained many yeast cells and fresh blood cells, and were traversed by narrow strands of persisting cerebral tissue. The yeast cells were small, round or oval, and frequently possessed buds or were arranged in short chains or small clusters (Fig. 7). Broad, clear haloes suggested the presence of abundant, soluble, capsular material. The yeast cells were observed also in the ventricles and meninges. There was no tissue reaction in the brain, and only a few polymorphonuclear leukocytes were in the meninges.

On the second day there was still no reaction in the brain, but polymorphonuclear leukocytes were in the infected areas of the meninges. Extension of the infection from the meninges into the underlying perivascular spaces was observed.

On the third day (Figs. 6 and 7) the masses of yeast cells in the brain were larger and still unattended by any inflammatory response except for a few small foci in relation to the choroid plexus or choroid fissure. The meninges were uniformly infected, and contained few polymorphonuclear leukocytes as compared with the large number of yeast cells. Although the inflammatory response was greater in the meninges than in the brain, it never approached the intensity observed in the subcutaneous tissues.

From the 6th to the 16th day, observations were made on animals which had received much smaller inoculative doses (1,300 to 1,300,000 yeast cells). Several of the animals had predominately meningeal involvement, with a more conspicuous inflammatory reaction. Mononuclear cells, phagocytized yeast cells, and early meningeal fibrosis were increasingly prominent. Small or large masses of yeasts with relatively few inflammatory cells were observed in the substance of the brain or filling dilated ventricles. There was no evidence of encapsulation of these foci, and alteration of surrounding tissue was minimal; but there was increasing phagocytosis of the yeast cells (Fig. 8).

All the doses of *C. neoformans* proved lethal when injected in the brain, and the survival period was inversely proportional to the dose (Text-fig. 1).



Text-figure 1. Mortality from experimental cryptococcosis. Groups of five mice received intracerebral (squares) or intraperitoneal (triangles) inoculation of *C. neoformans* in serial 10-fold dilutions.

Dissemination of infection occurred in all of these animals. Metastases were observed (in order of frequency) in liver, heart, lung, kidney, and spleen. Yeast cells were observed in the capillaries of these organs as early as the first day after inoculation. The liver was involved with greatest severity as well as greatest frequency. By the second day, the small foci of yeast cells in the hepatic sinusoids were infiltrated by polymorphonuclear leukocytes. On the third day these areas resembled miliary abscesses (Fig. 9). In most cases observed thereafter, the liver was studded with such lesions, and there was gradual replacement of polymorphonuclear leukocytes by macrophages

containing yeast cells. In several animals, the severe inflammation in the liver was in striking contrast to the minimal reaction in the brain (Figs. 6, 7, and 9). The degree of inflammatory response in the liver varied among the individual animals. It was generally much greater, however, than in the other organs.

Intraperitoneal Inoculation

Seventeen female mice of similar strain and average age were inoculated intraperitoneally with the same saline suspension used for the subcutaneous and intracerebral inoculations. In order to compare the lethality of intraperitoneal and intracerebral routes of inoculation, groups of five mice were inoculated with four serial decimal dilutions of this saline suspension.

On the first day after inoculation, no changes were detected in sections of liver, spleen, kidney, lung, heart, and brain. On the second and third days, there was a small amount of purulent exudate on the surfaces of the spleen and liver. Yeast cells were present in this exudate and in small numbers in the parenchyma of these organs.

On the 7th and 13th days, the liver showed miliary abscesses. Disseminated cerebral lesions showed much less inflammation than the hepatic lesions. A comparable difference in inflammatory response was noted previously when the brain was the primary site of inoculation.

After 2 weeks, the same progressive changes, as described previously, were observed in the lesions.

C. neoformans proved far less lethal by the intraperitoneal route than by the intracerebral route (confirming Emmons' data)⁴ and the mortality rate was not proportional to dosage (Text-fig. 1). Between 2 weeks and 5 months after inoculation, 23 animals died or were sacrificed. Cryptococcal infection in 12 animals was confirmed by cultures or histologic slides. The brain was involved in seven, the liver in six, and the heart, lungs, spleen, and kidneys in one animal with widely disseminated lesions, and the perirenal adipose tissue in one animal. The involvement of the liver was slight, and sometimes detected only by culture. Involvement of the brain was more frequent and usually took the form of large lesions that had caused motor signs during life. As in man, the cerebral lesions were the significant ones.

Two animals (sacrificed after 2 and 5 months) showed involvement of the muscles and subcutis of the abdominal wall, without other lesions. Histologically, both cases were characterized by a combined purulent and granulomatous reaction (Fig. 10). The granulomas contained multinucleated giant cells with yeast cells in their cyto-

plasm. The localization of these lesions was probably due to inadvertent intramuscular inoculation. The granulomatous response may be related to the site and chronicity of the infection.*

No evidence of infection was detected in cultures or sections of 11 animals. Nine of these were examined 5 months after inoculation.

Observations with Other Strains of C. neoformans

In order to determine whether the histopathology and course of the infection varied with the strain of *C. neoformans*, the preceding experiments were repeated with two other strains. Groups of eight female A albino mice, of an average age of 3 months, were inoculated with a saline suspension from a 5-day broth culture by the subcutaneous, intraperitoneal, or intracerebral routes. The doses were 8.2 million cells for strain 1499.62 and 7.3 million cells for strain 1499.57. The animals died or were sacrificed at various intervals; the survivors were sacrificed at 4 months.

After subcutaneous inoculation of strain 1499.62 the lesions appeared more slowly, grew larger, and lasted longer than comparable lesions produced by strain 1499.20. Strain 1499.57 produced even larger and longer-lasting lesions. Both strains produced an early histopathologic response characterized by formation of an abscess, as was noted for strain 1499.20, and all lesions ulcerated. Strain 1499.57 was different from the others in that the proliferation of yeast cells outstripped the inflammatory response by the sixth day. Older lesions showed phagocytosis of yeast cells, but minimal infiltration of polymorphonuclear and mononuclear inflammatory cells, and variable fibrosis and encapsulation (Fig. 11). The yeast cells were larger and had broad haloes, indicating copious quantities of capsular material. Despite this histopathologic difference, the lesions tended to heal and only one animal in each group showed dissemination to the brain.

No differences were noted following intracerebral inoculation with the two strains. All animals died within 5 days. The cerebral lesions showed the characteristic absence of inflammatory response in marked contrast to the subcutaneous lesions of equal age. There was dissemination of infection to all organs examined.

Following intraperitoneal inoculation, all of the animals died or were sacrificed within 2 months except for one in each group which had no histologic or cultural evidence of disease at 4 months. All

* The absence of such a distinct granulomatous response in the series of animals inoculated subcutaneously may be related to the rapid healing of these lesions.

other animals had disseminated disease. Again the brain was the site of predilection and the cerebral lesions were more numerous, larger, and showed much less inflammatory response than those in other organs. Figures 13 and 14 illustrate severe involvement of the brain approaching the "soap-bubble" appearance sometimes observed in human cases.

Subcutaneous Inoculations in Rats and Rabbits

The development of abscesses following subcutaneous inoculation in mice was a finding of sufficient interest to warrant confirmation in other species. Ten female albino rats of Wistar strain, averaging 130 gm. in weight, and four female New Zealand rabbits, averaging 1,760 gm. in weight, were inoculated subcutaneously with saline suspension of 25.6 million cells from a 4 day broth of *C. neoformans*, strain 1499.20. Local lesions developed in all animals. None showed evidence of systemic illness during life, and none showed histologic or cultural evidence of disseminated disease after death.

Five rats were sacrificed and studied histologically at intervals from the 4th to the 27th day. The development of well encapsulated abscesses followed by ulceration closely paralleled the results in mice. Multinucleated giant cells containing yeasts were more numerous than in lesions of mice by the 17th day. The lesions regressed and were undetectable on the 79th day when the remaining five rats were sacrificed.

The four rabbits were sacrificed between 4 and 27 days after inoculation. Ulceration was observed in only one rabbit, but in other respects the histopathologic lesions were the same as those of rats.

DISCUSSION

The most important result of this experiment was the difference in degree and type of inflammatory response to *C. neoformans* in various organs. The prompt outpouring of polymorphonuclear leukocytes in the subcutaneous tissues, followed by lymphocytes, plasma cells, macrophages, fibrosis, and encapsulation, was in striking contrast to the delayed, weak, and irregular reaction in the brain. The visceral organs were intermediate in reactivity.

The healing of subcutaneous infections with only occasional dissemination, the universal and prompt lethality of cerebral infections, and the intermediate reactivity of intraperitoneal infections probably were related directly to the characteristics of the inflammatory response. Preferential localization in the brain following intraperitoneal

inoculation can be interpreted as a lack of resistance to circulating yeast cells, based in turn on the minimal inflammatory response. The small size and many buds of the yeast cells in lesions of the brain indicate active growth in a favorable medium, while the larger size and fewer buds in subcutaneous abscesses probably are due to an unfavorable environment for growth.⁵ The similarity of the cerebral lesions in human and experimental cryptococcosis suggest that the same explanation may account for the predilection for the human central nervous system. In animals as in humans the lesions are not only more numerous in the brain than elsewhere, but are larger and more significant in producing symptoms and a fatal outcome.

Variability in the outcome of experimental inoculations is to be expected, based on differences in the dose, virulence, and strain of *C. neoformans*, and on differences in species, strain, age, and individual resistance of the experimental animals. Some authors have found disseminated disease following subcutaneous inoculation in the mouse,^{2,6} rat,² and guinea pig,^{1,2,6,7} while in other instances complete localization and healing of subcutaneous lesions have been recorded for the mouse,⁸ guinea pig,² dog,⁹ and monkey.^{9,10} Under the conditions of our experiments, localization and healing of subcutaneous infections have been the rule in the mouse, rat, and rabbit. Instances of healing of human infections localized to skin, lung, or bone have been recorded.^{2,3}

Dissemination of infection following subcutaneous inoculation occurred in two of our mice even though the skin lesions showed partial and complete healing, and similar observations are cited by Freeman.¹ This may explain the occurrence of disseminated cryptococcosis in humans despite the absence of a detectable portal of entry. In view of the isolation of cryptococci from normal human skin¹¹ and from soil,¹² the skin as well as lung must be considered a possible portal of entry.

The formation of true abscesses has not been considered common in human or experimental cryptococcosis, but it has been recorded in a few instances.¹³⁻¹⁷ The explanation is probably that early lesions have not been studied histologically. Baker and Haugen¹⁸ found numerous polymorphonuclear neutrophils with a few abscesses in five of 26 human cases. They suggested that hypersensitivity with increased tissue destruction might be the cause of the pyogenic responses. They also stated that "*C. neoformans* is a bland and inert agent when it first invades the body, and it produces no perceptible necrosis or inflammation in adjacent tissues. . . . There is no indication of a

primary neutrophilic response." Clearly, these suggestions are not applicable to the pyogenic response observed immediately following all subcutaneous inoculations under the particular conditions of our experiment.

Attempts to Alter the Course of Experimental Cryptococcosis

C. neoformans usually is considered to have little immunogenic activity, although useful antisera have been prepared in rabbits by some workers.^{19,20} Hoff²¹ attempted to produce active immunity in mice by injecting heat-killed cryptococcal cells, but he found only slight prolongation of life following intraperitoneal challenge. In view of the usually self-limited character of subcutaneous infections under the experimental conditions described here, we attempted to detect evidence of active immunity during the healing phase of subcutaneous lesions.

Sixteen female C57-black mice, of an average age of 4 months, were inoculated subcutaneously with 15.4 million cells of *C. neoformans*, strain 1499.20. Sixteen control mice received sterile saline injections. On the 14th day, when the subcutaneous lesions were regressing, all animals were challenged by the injection into the brain of 190,000 cells of the same strain. By the 13th day after challenge, all mice of the control series and 14 of the experimental series were dead. The survival of two mice of the experimental series to the 20th day does not, in our opinion, constitute a significant difference. Under the conditions of this experiment, we could not demonstrate active immunity associated with healing subcutaneous lesions.

It proved easier to decrease, rather than increase, the resistance of mice to experimental cryptococcosis. Cortisone is known to decrease inflammatory response.²² Cortisone and radiation are reported to act synergistically in increasing susceptibility to fungi and other infectious agents.²³ Although the possibility of a relation between human cryptococcosis and cortisone therapy has been raised,^{24,25} there is as yet little evidence for this thesis.¹⁷ Increased dissemination of experimental cryptococcosis due to cortisone has been reported following intraperitoneal inoculation of rats,²⁶ while only minimal enhancement occurs following intraperitoneal infection of mice.²⁷ In view of the usually localized nature of subcutaneous infection in our experiments, it was of interest to determine whether cortisone would cause dissemination.

Five groups of six female A albino mice (average age, 2½ months) were inoculated subcutaneously with 9.6 million cells of *C. neoformans*, strain 1499.20. One group was untreated, and served as a

control. The second group was subjected to daily manual trauma of the subcutaneous lesions. The third group received 500 r. of whole body x-radiation 20 hours prior to inoculation. The fourth group received 2.5 mg. of cortisone acetate in 0.1 cc. of saline solution intraperitoneally, 1½ hours before inoculation. The dose was repeated after 1 week. The fifth group received both cortisone and x-radiation in dosages as detailed above. All surviving animals were sacrificed after 3 weeks and evidence of disseminated disease sought by cultures and histologic sections.

The control mice developed subcutaneous lesions which ulcerated and regressed as usual. When they were sacrificed, all appeared healthy and the lesions had almost completely healed. There was no evidence of dissemination except for a positive culture from the liver of one animal. The lesions which had been traumatized showed ulceration, healed more slowly, and a few yeast cells still were present in the healing abscesses. Granulomatous inflammation with multinucleated giant cells was more conspicuous than usual around the abscesses. There was, however, no dissemination of infection.

The appearance of the lesions was the same in the irradiated group as in the controls, but healing was a little slower. Two of the animals that died during the experiment showed impaired inflammatory response in the subcutis and widely disseminated lesions. Cultures of the lungs of one of the four remaining mice taken at the termination of the experiment revealed cryptococci.

Cortisone had a much more dramatic effect on the course of subcutaneous cryptococcal lesions. The appearance of palpable lesions was delayed 3 to 5 days after inoculation. The early lesions remained much smaller than those of the controls, but they grew progressively, and after 13 days were much larger than in the control mice. Only one lesion ulcerated despite the large size. Dissemination occurred in all animals, and three of them died before the end of the experiment. Sections of the subcutaneous lesions showed poor or absent inflammatory response, and absence of encapsulation. As noted in other experiments, the brain was the site of predilection and usually had the largest metastatic foci. The delayed appearance, small size of early lesions, and absence of ulceration could be related to the paucity or absence of polymorphonuclear leukocytes. The large size of late subcutaneous lesions was due to the rapid, unbridled proliferation of yeast cells.

The results with combined cortisone and x-radiation paralleled those with cortisone alone, except that all animals died by the 12th day. Disseminated disease was present in all animals examined, but

it was less prominent and widespread, with little involvement of brain, probably due to the early demise of the animals. Again, lack of inflammation was notable in the subcutaneous lesions (Fig. 12).

SUMMARY

Experimental cryptococcosis was produced by subcutaneous, intracerebral, and intraperitoneal inoculations using three strains of *Cryptococcus neoformans*. The subcutaneous lesions were characterized by early leukocytic response, abscess formation, encapsulation, healing, and only occasional instances of dissemination. Lesions of the brain were characterized by proliferation of the yeast cells to form large lesions with minimal inflammation and with universal dissemination. More inflammation was noted in the meninges, but here, too, the reaction was weak and irregular.

Intraperitoneal inoculations were attended by an intermediate degree of inflammatory response (usually most marked in the liver) and were also intermediate with respect to dissemination and lethality. The predilection for the brain in disseminated infection was thought to be directly related to the minimal inflammatory response in cerebral tissue.

The healing subcutaneous cryptococcal abscesses were not associated with active immunity against subsequent intracerebral challenge.

The inflammatory response to subcutaneous inoculation of *C. neoformans* was abolished by cortisone, and, to a lesser extent, by x-radiation. This was accompanied by an increased dissemination of the infection.

REFERENCES

1. Freeman, W. Torula infection of the central nervous system. *J. f. Psychol. u. Neurol.*, 1931, **43**, 236-345.
2. Cox, L. B., and Tolhurst, J. C. Human Torulosis. Melbourne University Press, Carlton, Victoria, Australia, 1946, 149 pp.
3. Littman, M. L., and Zimmerman, L. E. Cryptococcosis. Grune & Stratton, New York, 1956, 205 pp.
4. Emmons, C. W. *Cryptococcus neoformans* strains from a severe outbreak of bovine mastitis. *Mycopath. et mycol. appl.*, 1952, **6**, 231-234.
5. Longmire, W. P., Jr., and Goodwin, T. C. Generalized Torula infection. Case report and review with observations on pathogenesis. *Bull. Johns Hopkins Hosp.*, 1939, **64**, 22-43.
6. Binford, C. H. Torulosis of the central nervous system. Review of recent literature and report of a case. *Am. J. Clin. Path.*, 1941, **11**, 242-251.
7. Bureau, Y.; Barrière, H., and Trichereau, R. La torulose cutanée. *Ann. de dermat. et syph.*, 1955, **82**, 484-509.
8. Wade, L. J., and Stevenson, L. D. Torula infection. *Yale J. Biol. & Med.*, 1940-41, **13**, 467-476.

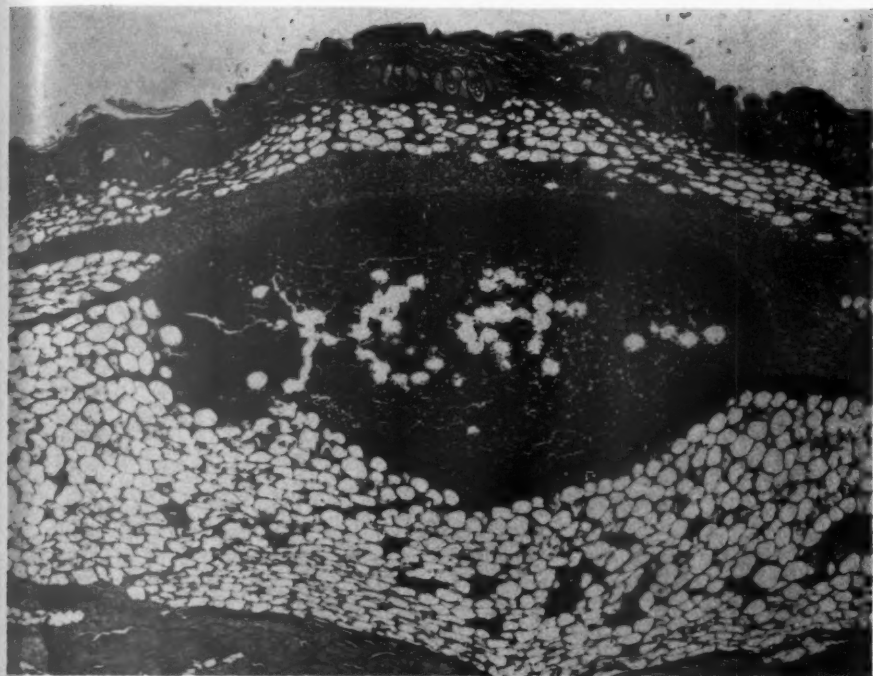
9. Debré, R.; Lamy, M.; Leblois, C.; Nick, J.; Grumbach; and Normand, E. Sur la torulose. Etude clinique et expérimentale. (A propos d'un cas observé chez un enfant atteint de lymphogranulomatose maligne.) *Ann. paediat.*, 1947, **168**, 1-33.
10. Weidman, F. D. Cutaneous torulosis. The identification of yeast cells in general in histologic sections. *South. M. J.*, 1933, **26**, 851-863.
11. Benham, R. W. Cryptococci—their identification by morphology and by serology. *J. Infect. Dis.*, 1935, **57**, 255-274.
12. Emmons, C. W. Isolation of *Cryptococcus neoformans* from soil. *J. Bact.*, 1951, **62**, 685-690.
13. Nichols, E. H. The relation of blastomycetes to cancer. *J. M. Research*, 1902, **7**, 312-359.
14. Brewer, G. E., and Wood, F. C. Blastomycosis of the spine. Double lesion: two operations: recovery. *Ann. Surg.*, 1908, **48**, 889-896.
15. McGehee, J. L., and Michelson, I. D. Torula infection in man, report of a case. *Surg., Gynec. & Obst.*, 1926, **42**, 803-808.
16. Symmers, W. St. C. Torulosis; case mimicking Hodgkin's disease and rodent ulcer and presumed case of pulmonary torulosis with acute dissemination. *Lancet*, 1953, **2**, 1068-1074.
17. Zimmerman, L. E., and Rappaport, H. Occurrence of cryptococcosis in patients with malignant disease of reticuloendothelial system. *Am. J. Clin. Path.*, 1954, **24**, 1050-1072.
18. Baker, R. D., and Haugen, R. K. Tissue changes and tissue diagnosis in cryptococcosis. A study of 26 cases. *Am. J. Clin. Path.*, 1955, **25**, 14-24.
19. Evans, E. E. An immunologic comparison of twelve strains of *Cryptococcus neoformans* (*Torula histolytica*). *Proc. Soc. Exper. Biol. & Med.*, 1949, **71**, 644-646.
20. Neill, J. M.; Abrahams, I., and Kapros, C. E. A comparison of the immunogenicity of weakly encapsulated and of strongly encapsulated strains of *Cryptococcus neoformans* (*Torula histolytica*). *J. Bact.*, 1950, **59**, 263-275.
21. Hoff, C. L. Immunity studies of *Cryptococcus hominis* (*Torula histolytica*) in mice. *J. Lab. & Clin. Med.*, 1942, **27**, 751-754.
22. Spain, D. M.; Molomut, N., and Haber, A. Studies of the cortisone effects on the inflammatory response. I. Alterations of the histopathology of chemically induced inflammation. *J. Lab. & Clin. Med.*, 1952, **39**, 383-389.
23. Syverton, J. T.; Werder, A. A.; Friedman, J.; Roth, F. J., Jr.; Graham, A. B., and Mira, O. J. Cortisone and roentgen radiation in combination as synergistic agents for production of lethal infections. *Proc. Soc. Exper. Biol. & Med.*, 1952, **80**, 123-128.
24. Lauzé, S. Infection à Torula chez un individu traité aux antibiotiques et à la cortisone. *Union méd. du Canada*, 1952, **81**, 935-940.
25. Linden, I. H., and Steffen, C. G. Pulmonary cryptococcosis. *Am. Rev. Tuberc.*, 1954, **69**, 116-120.
26. Königsbauer, H. Über die Wirkung von Corton auf die experimentelle Torulose und Chromoblastomykose. *Zentralbl. f. Bakt.*, 1954, **160**, 637-643.
27. Truant, J. P., and Tesluk, H. The effect of cortisone upon experimental cryptococcosis. *Bact. Proc.*, 1956, 87.

[Illustrations follow]

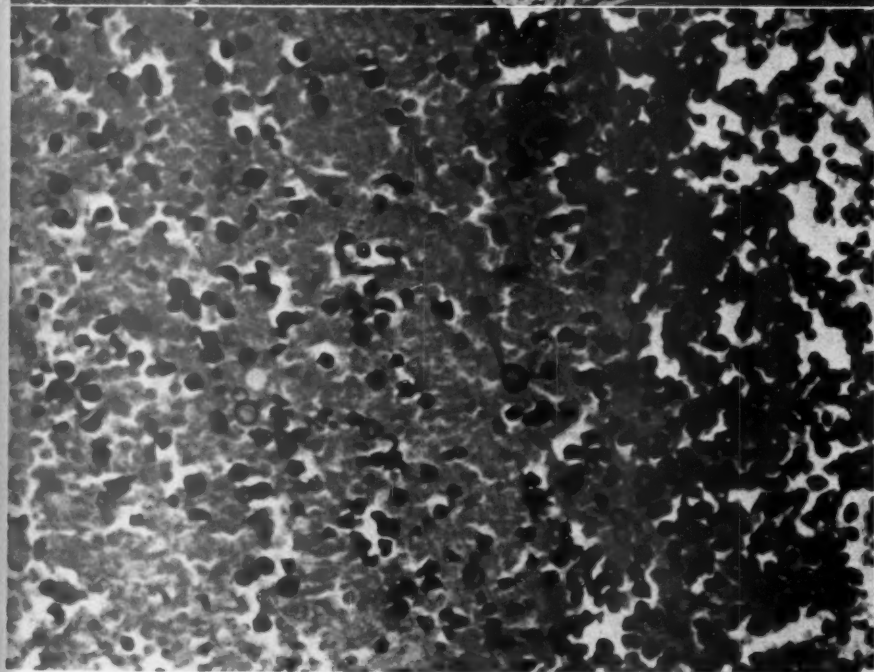
LEGENDS FOR FIGURES

FIG. 1. Abscess in subcutis 2 days after inoculation of *Cryptococcus neoformans*, strain 1499.20. Hematoxylin and eosin stain. $\times 45$.

FIG. 2. Cryptococcal cells in center of abscess, 3 days after inoculation. The faintly stained cells are polymorphonuclear leukocytes. Periodic acid-Schiff (PAS) stain. $\times 440$.



1



2

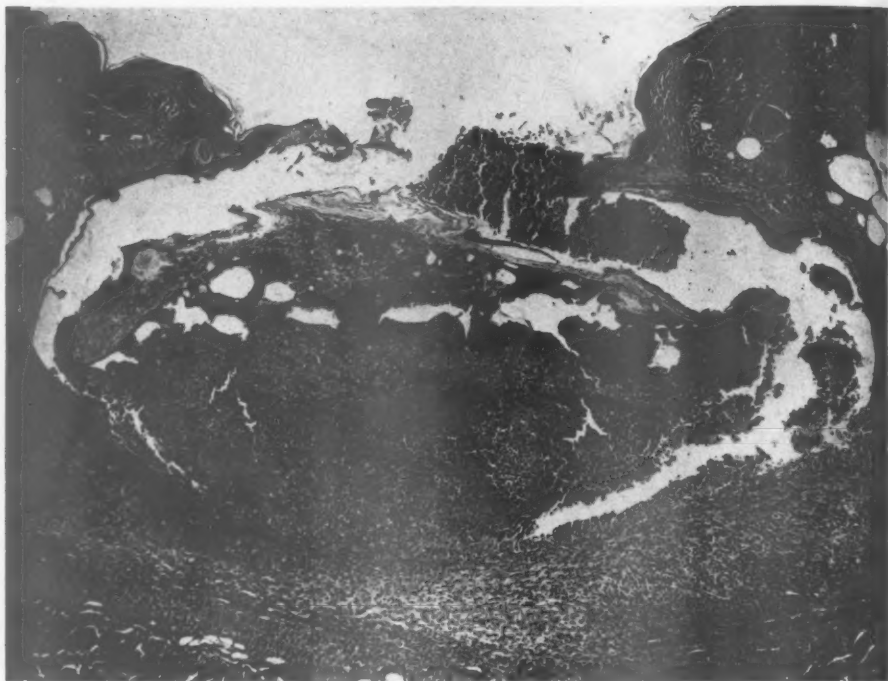
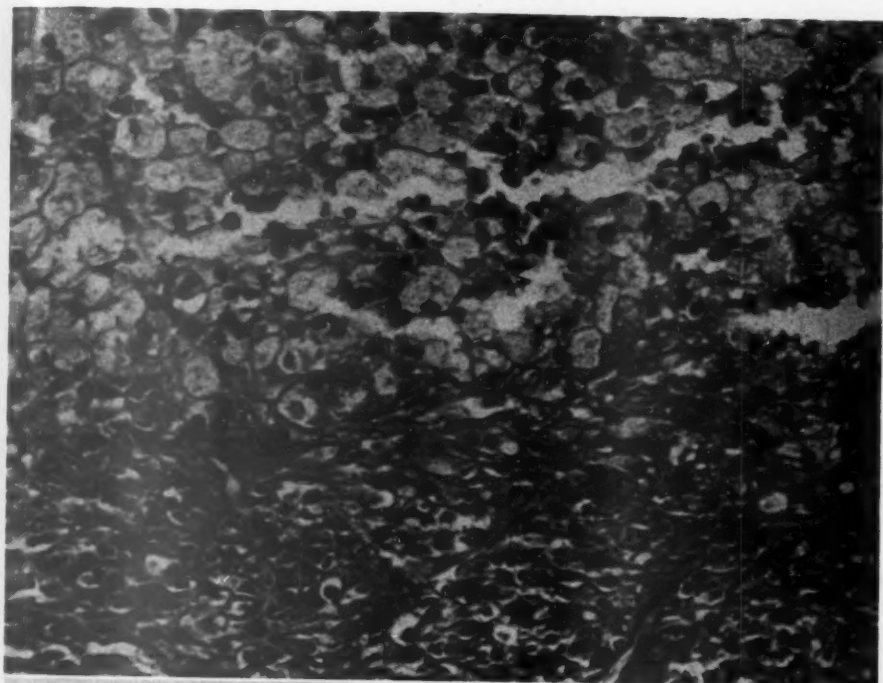


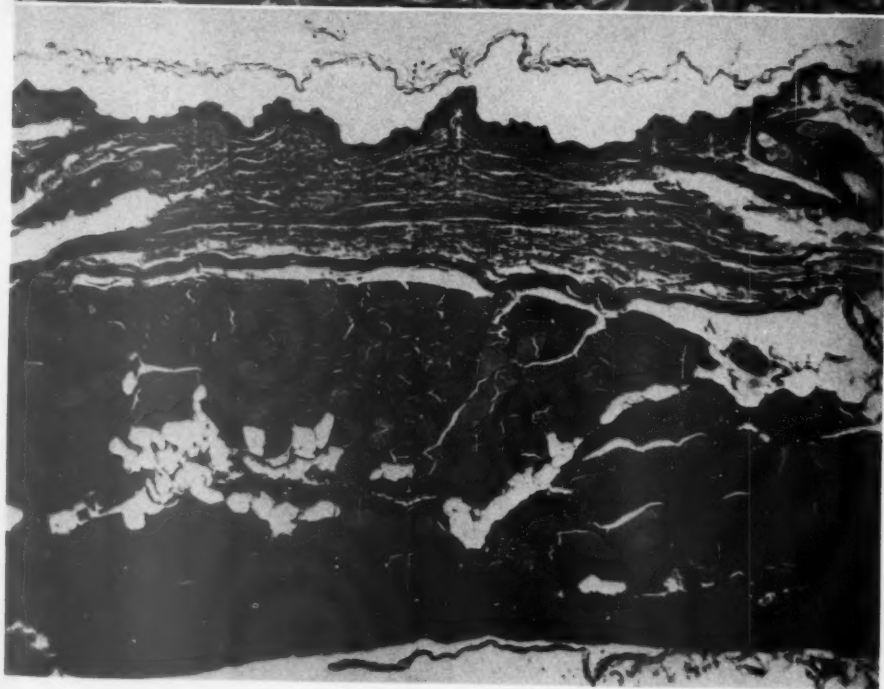
FIG. 3. Ulceration with separation of necrotic skin and contents of abscess, 7 days after inoculation. There is downward proliferation of the epidermis and walling off of the base of the abscess. Hematoxylin and eosin stain. $\times 61$.

FIG. 4. Foamy macrophages and polymorphonuclear leukocytes in the center of the lesion (above) with surrounding wall of proliferated connective tissue infiltrated by lymphocytes and plasma cells (below), 20 days after inoculation. Hematoxylin and eosin stain. $\times 440$.

FIG. 5. Fibrosis of dermis and subcutis, 41 days after inoculation. Hematoxylin and eosin stain. $\times 120$.



4



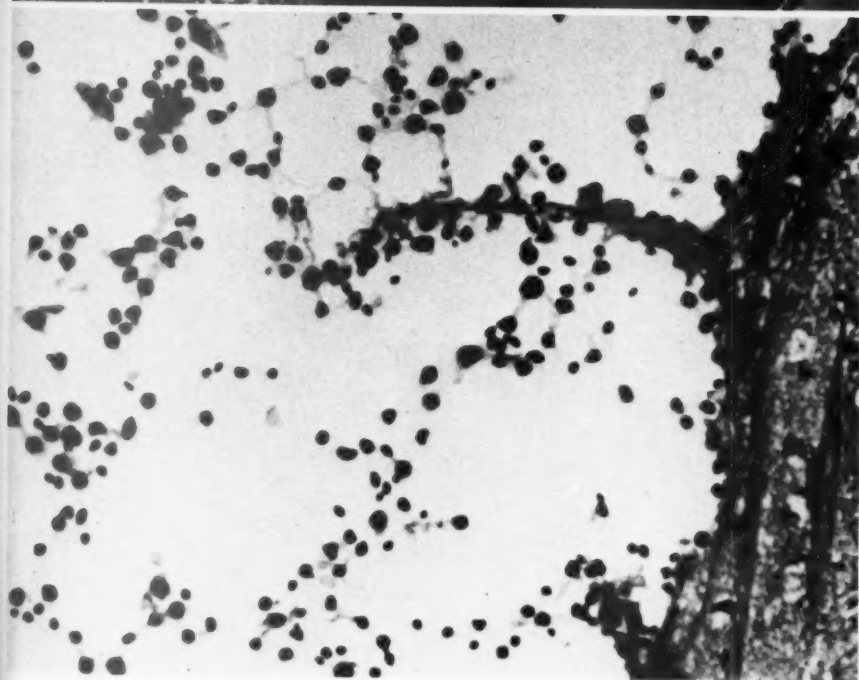
5

FIG. 6. Lesions of brain and meninges, 3 days after intracerebral inoculation. The clear areas contain many yeast cells (small faint dots), and fresh blood (dark material in center), but there is no inflammatory response. Hematoxylin and eosin stain. $\times 65$.

FIG. 7. Edge of lesion of brain depicted in Figure 6. The darkly stained cryptococcal cells show innumerable buds and the clear areas probably represent soluble capsular polysaccharide. There is no inflammatory response. PAS stain. $\times 440$.



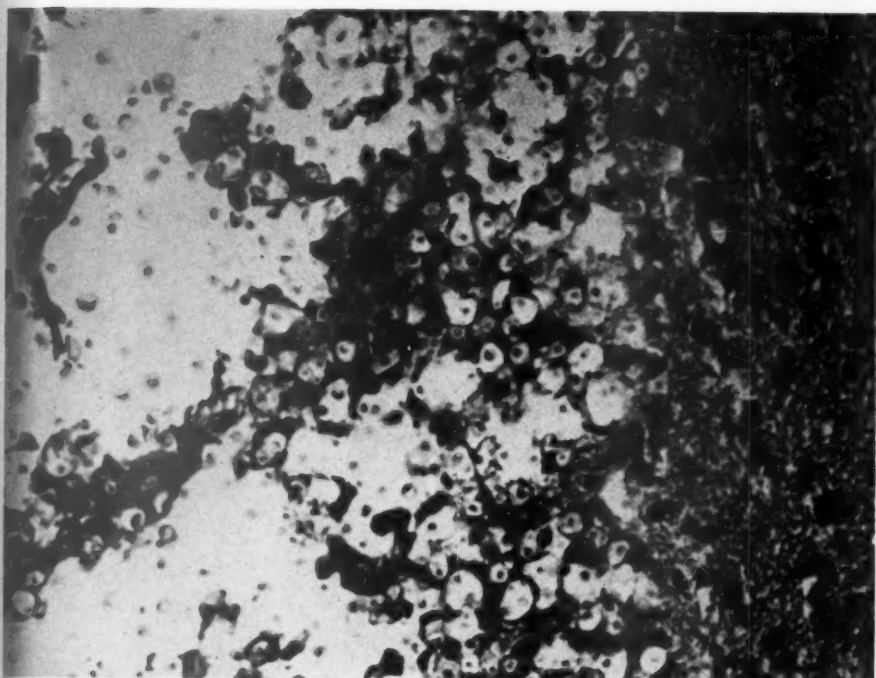
6



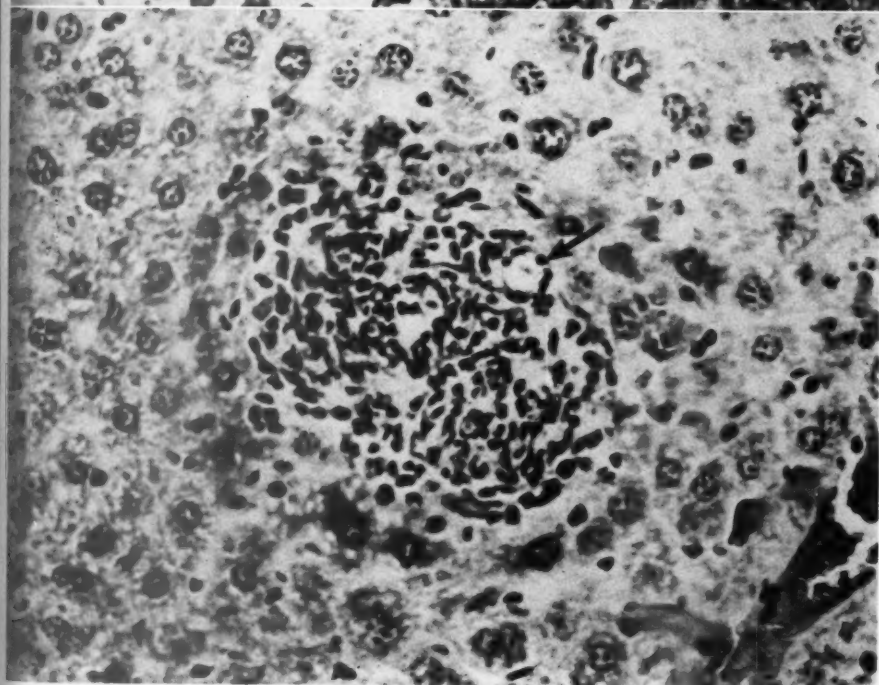
7

- FIG. 8. Edge of lesion of brain, 16 days after intracerebral inoculation. Many of the faintly stained cryptococci are within the cytoplasm of darkly stained phagocytes. There is no encapsulation of the lesion. Hematoxylin and eosin stain. $\times 440$.
- FIG. 9. Miliary abscess in liver, 3 days after intracerebral inoculation (same animal as that from which Figures 6 and 7 were taken). Yeast cells are few and faintly stained (arrow). Hematoxylin and eosin stain. $\times 440$.





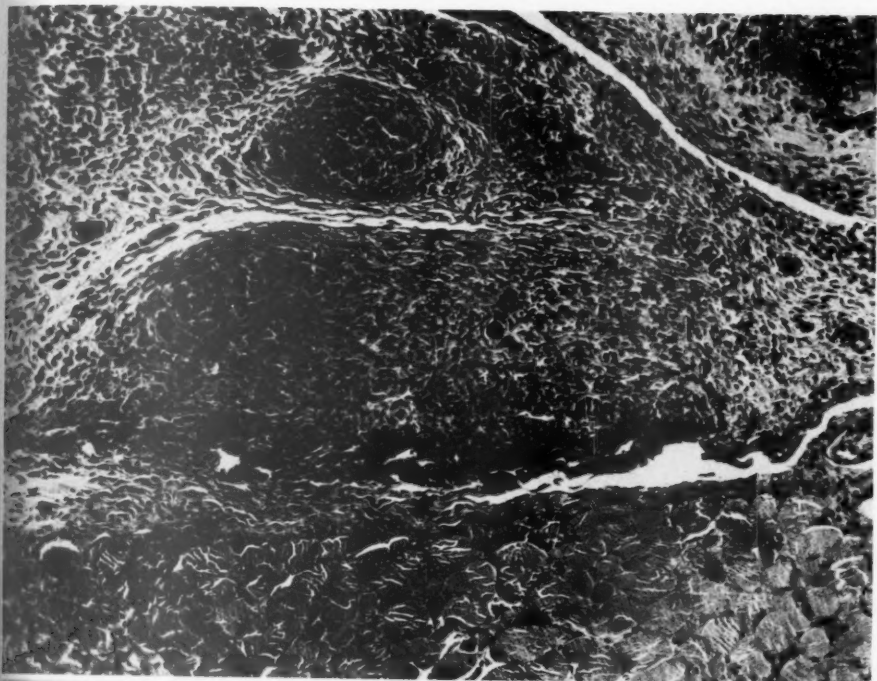
8



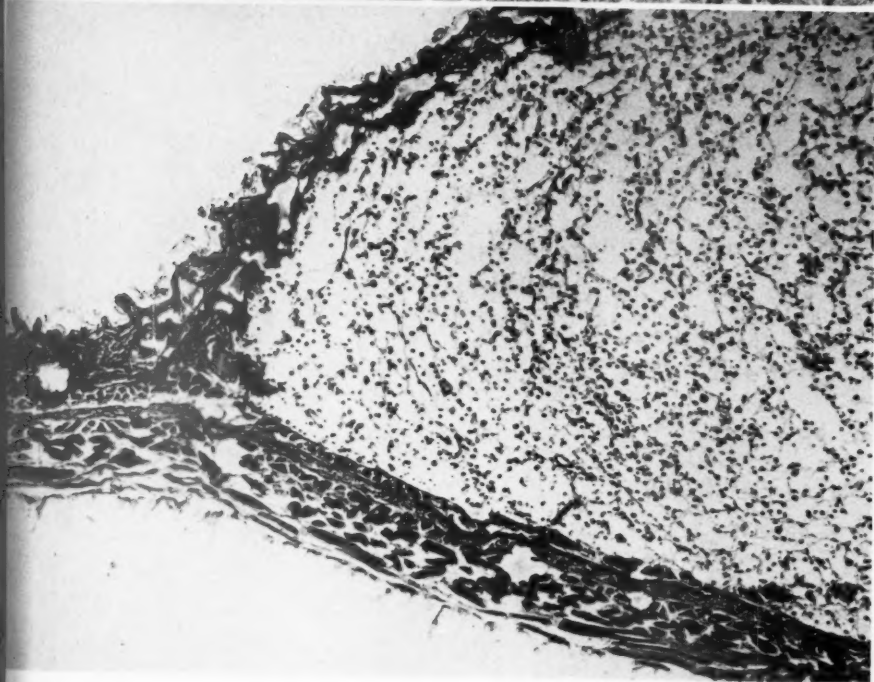
9

FIG. 10. Granulomatous reaction with numerous giant cells in abdominal wall, probably due to inadvertent intramuscular inoculation. Hematoxylin and eosin stain. $\times 120$.

FIG. 11. Subcutaneous lesion 15 days after inoculation with *C. neoformans*, strain 1499.57. The lesion is large and poorly encapsulated, and contains innumerable darkly stained yeast cells with but few inflammatory cells. PAS stain. $\times 65$.



10



11

12

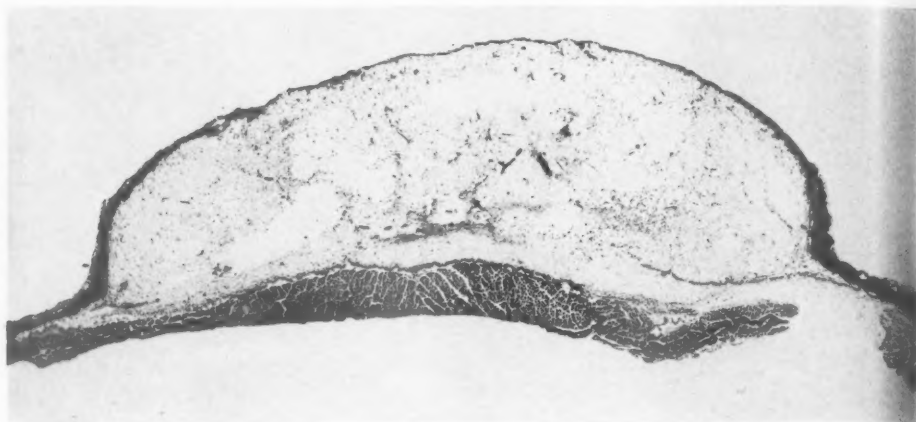
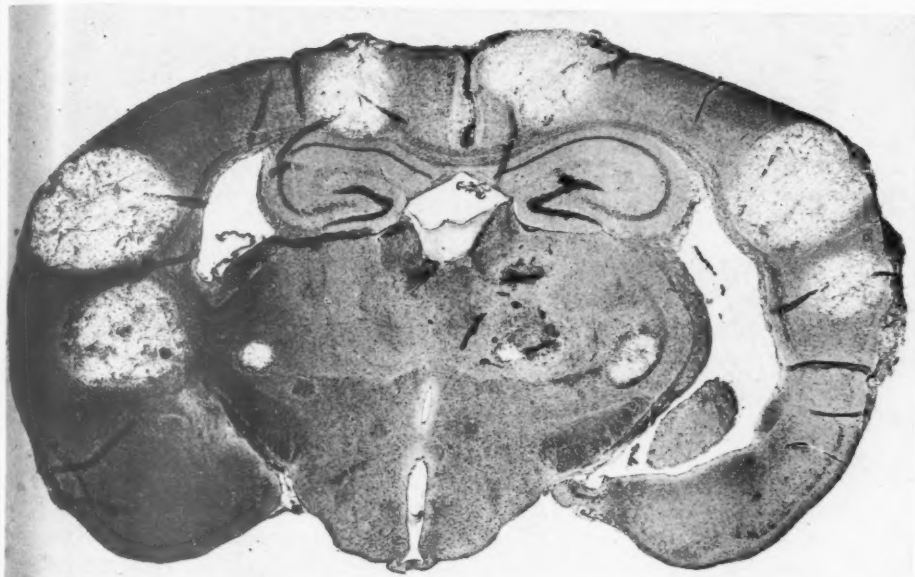
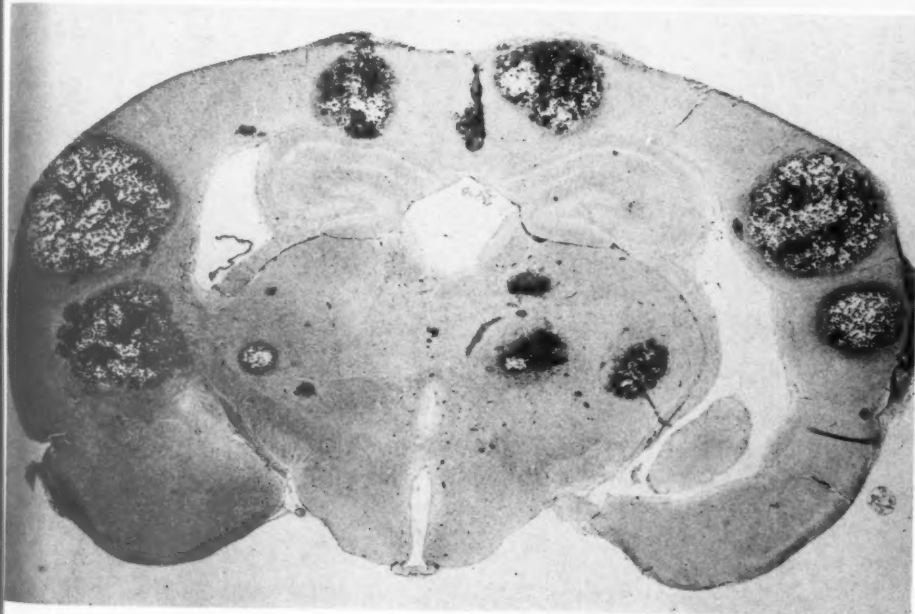


FIG. 12. Subcutaneous lesion, 12 days after inoculation of *C. neoformans*, strain 1499.20, in a mouse treated with cortisone and x-radiation. There is no inflammation or encapsulation, and no ulceration despite the large size of the lesion. Hematoxylin and eosin stain. $\times 15$.

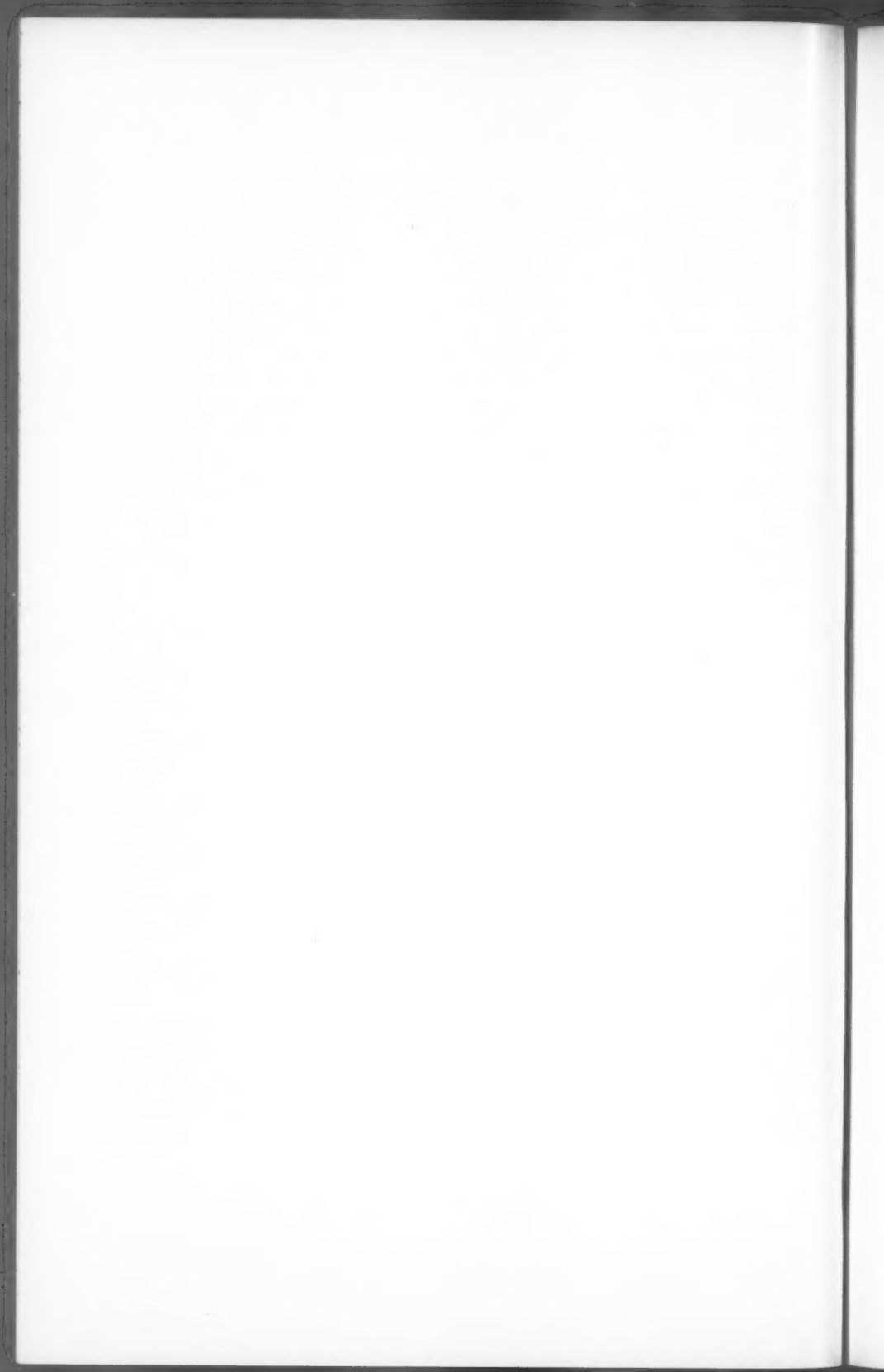
FIGS. 13 and 14. Severe involvement of brain, 29 days after intraperitoneal inoculation with *C. neoformans*, strain 1499.62. The lesions are pale in the section stained by hematoxylin and eosin (Fig. 13) because of the large, clear, capsular haloes and the minimal inflammatory response. They are dark in the section colored by the PAS stain (Fig. 14) because of the innumerable darkly stained yeast cells. $\times 18$.



13



14



MADUROMYCOTIC MYCETOMAS IN ANIMALS
CURVULARIA GENICULATA AS AN ETIOLOGIC AGENT*

CHARLES H. BRIDGES, D.V.M.

*From the School of Veterinary Medicine, A. & M.
College of Texas, College Station, Texas*

This report concerns the occurrence in three dogs and one horse of mycetomas of the type known in man as maduromycosis. These are mycotic granulomas that may be caused by any one of several fungi of the classes Ascomycetes and Fungi Imperfecti. They occur in the tissues as definite microcolonies or grains which are composed of large segmented mycelial filaments possessing well defined walls, and usually chlamydospores or other spores.¹ The following brief review of the literature gives the historical background which has established this as a definite pathologic entity.

In 1842, Gill² of Madura, India, described a pathologic process in the foot of a man which was characterized by marked deformity and fungoid excrescences with an offensive ichorous discharge. The disease was represented by a granulation tissue resembling fibrocartilage, and destroyed joints, cartilages, and ligaments. In 1846, Colebrook,³ who succeeded Gill at Madura, confirmed these observations, and stated that this disease was commonly known in some parts of India as "Madura foot." In 1845, Godfrey⁴ described the occurrence in a man's foot of a similar condition which had a considerable black deposit resembling small fragments of coal. Vandyke Carter⁵ established the fungal nature of Madura foot for which he proposed the term mycetoma in 1860. He differentiated forms with ochroid or pale yellow grains and forms with melanoid or black grains, but considered them to be the same pathologic process.

Boyce and Surveyor,⁶ in 1894, proved that the fungi in the black and yellow grains were different and thereby established the two main divisions of mycetomas which Pinoy,⁷ in 1913, called actinomycosis and true mycetomas. The true mycetomas were caused by the higher fungi or Ascomycetes and Fungi Imperfecti. Chalmers and Archibald⁸ introduced the term maduromycosis to differentiate the mycetomas of Vandyke Carter and the true mycetomas of Pinoy. Their definition of maduromycosis was as follows: "those forms of Mycetoma with grains composed of large segmented mycelial filaments possessing well defined walls, and usually chlamydospores." Actinomycosis was defined as "those forms of Mycetoma with grains com-

* Received for publication, July 13, 1956.

posed of very fine non-segmented mycelial filaments, in which usually the walls are not clearly defined from the contents, and in which chlamydospores are absent." The term grain, as used by these authors, refers to the microcolonies in the mycetomas. They are found embedded in the tissue or in the discharge from it.⁹ Chalmers and Archibald attempted to divide the maduromycoses according to the color of the grains, but such a classification has been found to be inaccurate because fungi of different genera and classes produce grains of the same color and at least one, *Sterigmatocystis nidulans*,⁷ can produce both white and black grains.

The genera of fungi which have been reported to cause maduromycosis¹⁰ and the color of the grains produced by them¹¹ are as follows: *Allescheria Boydii* Shear, 1921—white grains; *Aspergillus Bouffardi* Brumpt, 1906—black grains; *Sterigmatocystis nidulans* var. *Nicollei*, Pinoy, 1906—black grains and white grains (experimental disease in the pigeon⁷); *Penicillium mycetogenum* Mantelli and Negri, 1915—black grains; *Madurella* (10 species)—black grains; *Indiella* (3 species)—white grains; *Monosporium apiospermum* Saccardo, 1911—white grains; *Cephalosporium* (3 species)—yellowish white grains; and *Phialophora Jeanselmei* (Langeron) Emmons, 1945—black grains.

Conant *et al.*¹⁰ considered that *Monosporium apiospermum* Saccardo, 1911, *Allescheria Boydii* Shear, 1921, and *Monosporium* (*Scedosporium*) *sclerotiale* Pepere, 1914, are synonyms, and that *Monosporium apiospermum* is the imperfect or asexual form of *Allescheria Boydii*. Brumpt¹¹ described *Monosporium* (*Scedosporium*) *sclerotiale* Pepere, 1914, as producing black grains whereas lesions caused by *Monosporium apiospermum* and *Allescheria Boydii* contain white grains.¹⁰ If these organisms are the same except for slight biologic variation, then it is of interest to note that color variation of grains occurs primarily in genera of the Ascomycetes.

As mentioned, Chalmers and Archibald⁸ defined the maduromycoses as those forms of mycetomas with grains composed of large segmented mycelial filaments possessing well defined walls, and usually chlamydospores. Puestow¹² found a case in a young girl caused by *Sterigmatocystis* (*Aspergillus*) *nidulans* in which no hyphae or chlamydospores were present. The grains consisted of small spores and budding bodies only, so he proposed that the definition for maduromycosis should be amended to read "with or without hyphae or chlamydospores." He agreed that the point was being stretched to designate as grains the colonies which he found, in the sense of the accepted definition. Some grains caused by *Aspergillus Bouffardi*¹¹ contain aspergillar heads as well as chlamydospores.

Thus, from a pathologic process affecting the feet of man which was described as Madura foot before the advent of serious microscopic study of tissues or fungi, we have come to recognize maduromycosis, the subject of this paper, as well as actinomycosis and nocardiosis. Maduromycotic lesions also occur in other parts of the body, such as the neck, head, and groin.^{10,12} One diagnosis of maduromycosis of the central nervous system in a woman was based on the finding of granulomatous meningitis and the repeated isolation of *Allescheria Boydii* from the spinal fluid.¹³ The microcolonies were not demonstrated. Not only do twenty-four species of ten genera and two classes of fungi participate as etiologic agents in maduromycosis, but several of these organisms have been found to cause other infectious processes; viz., *Allescheria Boydii* in otomycosis and septicemia of man.¹⁰

The pathologic process in maduromycosis deserves recognition as an entity regardless of the diverse etiologic agents involved, because it follows the course of a chronic debilitating infectious process which resists practically all therapy except complete surgical excision of the tissue containing the microcolonies. It is quite important to differentiate nocardiosis and actinomycosis from it (also, botriomycosis or staphylococcal granuloma), because the organisms of the latter can be controlled quite successfully with antibiotics. Also, maduromycosis should be differentiated from such mycotic infections as mucormycosis, which are most commonly associated with important predisposing factors such as diabetes mellitus, carcinoma, and antibiotic therapy.¹⁴ Mucormycosis is diagnosed on histopathologic examination by the presence of very wide (6 to 50 μ^{10}), branching, coenocytic hyphae in the tissues with no definite formation of grains. Occasionally, septa may be seen.¹⁵ The hyphae extend indiscriminately through the tissues and are surrounded on all sides by inflammatory tissue.

When pathogenic aspergilli invade the deep tissues, they produce considerable necrosis. The hyphae, 2 to 5 μ in diameter, also run indiscriminately through necrotic and granulomatous tissue. Occasionally, they induce small granulomas which consist of foci of purulent exudate and hyphae surrounded by definite coronas of epithelioid cells and granulation tissue. The bulk of the central mass is exudate and necrotic debris. Although aspergilli do not produce spores in the deeply seated lesions, they may do so when the hyphae reach an adequate amount of oxygen such as occurs in the bronchioles of the lungs or the air sacs of birds. In the presence of oxygen they produce conidia and even typical aspergillar conidiophores. Except for the

narrower hyphae which contain numerous septa, aspergillosis might be confused with mucormycosis on histologic examination of the lesions.

Generalized visceral infections with *Candida albicans* produce microscopic lesions resembling those of aspergillosis, except that the organisms form short hyphae and blastospores which occur singly and in chains. At the present time the disease in animals is essentially an experimental one.

Only two reports of maduromycotic mycetomas in animals have been found. Robinson¹⁶ submitted a case to the 1952 Seminar of the American College of Veterinary Pathologists. The affected animal, a 5-year-old pointer from Florida, had had a mycetoma between the toes of the right front foot for over 2 years. The infection had metastasized to the regional lymph nodes. Seibold¹⁷ reported one case involving the left front foot of a 3-year-old greyhound from Florida. The microcolonies or grains in these cases were dark brown in thin sections. Seibold cited Riser¹⁸ to the effect that aspergilli caused such lesions in the feet of animals, but a review of Riser's statement reveals only vague discussion of mycetomas, including those of man and animals. Riser used the term chromoblastomycosis and Madura foot synonymously and referred to Robinson's case as chromoblastomycosis. Georg¹⁹ identified a fungus, supposedly isolated from a mycetoma in a dog's foot, as *Helminthosporium* sp. Akün²⁰ reported a mycetoma with pigmented microcolonies in the nasal cavity of a Turkish cavalry horse, and he classified the lesion as chromoblastomycosis. Although the photomicrographs in Akün's report show no hyphae, the colonial configuration is quite similar to many of those in case 3 of this report in which the mycelial component was absent. His case also resembles one in a man reported by Symmers and Sporer²¹ in which no hyphae were present. Quite similar microcolonies are in evidence in Robinson's case. Also, Akün's photomicrographs do not adequately demonstrate the septate bodies which are so characteristic in lesions of chromoblastomycosis in man.¹⁰ Such evidence, although based entirely on Akün's report, suggests that his case might have been similar to those reported here.

REPORT OF CASES

*Case 1**

A 4-year-old Walker hound was submitted for clinical examination in September, 1954, with a tumefaction of the interdigital spaces of the right front foot. A diagnosis of mycetoma with dark brown microcolonies was made by histopathologic examination. Subsequent ther-

* This animal was submitted for pathologic examination by Dr. O. E. Bockhorn, Brenham, Texas.

apy brought about remission of the tumefaction, but this recurred. Re-examination in October, 1955, revealed marked swelling of the same foot with many open fistulas from which small (0.5 to 1.5 mm.) microcolonies of a black fungus could be expressed (Fig. 4). The tumefaction involved all of the interdigital spaces and the tissue between the foot pads, up to and including the first digit (Fig. 1). Small fistulas were present in the metacarpal pad. Also, at the time of this second examination the left rear foot had a flattened tumefaction between digits 3 and 4 and this extended into the tissues on the plantar surface between the carpal and metacarpal pads (Fig. 2). The skin over the lesion was dark brown. The cut surface of a specimen taken for biopsy revealed many black foci, each surrounded by separate islands of yellowish tissue which in turn were separated from each other by thin trabeculae of white fibrous tissue (Fig. 3). The left front foot had a much smaller lesion containing similar grains of fungus, which were black to dark brown.

The microcolonies of fungus consisted of a central core of sparse, lightly pigmented, branching, septate hyphae, 3 to 6 μ in diameter, surrounded by a thick corona of large, deep brown chlamydospores, 12 to 25 μ in diameter. A few non-pigmented, bud-like projections extended outwardly from the row of chlamydospores. The hyphae, which were embedded in an albuminous fluid, had numerous swellings indicative of early formation of chlamydospores (Fig. 10). Most of the non-pigmented hyphae stained easily with hematoxylin whereas the pigment of the brown portions overshadowed any stain. In some areas the corona of chlamydospores was incomplete and formed horse-shoe and scroll-like configurations (Fig. 6).

Immediately surrounding many microcolonies was a network of fibers which was invaded by macrophages and neutrophils. These fibers stained quite basophilic in some areas and acidophilic in others with hematoxylin and eosin and were black in sections stained by Wilder's reticulum stain.²² Bands of this tissue from opposite sides of a colony often joined to extend outwardly in a double column from the colony into the adjacent granulomatous tissue. A few neutrophils and macrophages were scattered about in the center and around the periphery of the colonies, and surrounding the colonies was a very vascular granulomatous tissue consisting of many macrophages, a few lymphocytes, neutrophils, plasma cells, and strands of collagenous tissue. Many macrophages contained brown pigments, some of which were positive for iron as determined by the Prussian blue reaction, and others stained a reddish purple with the periodic acid-Schiff (PAS) technique.

An occasional colony was surrounded by foreign body giant cells with minimal numbers of neutrophils. These colonies appeared to be degenerating. Scattered throughout the granulomatous tissue surrounding the fungus were spherical to pyriform acidophilic bodies, 8 to 10 μ in diameter, all within macrophages or small multinucleated giant cells.

The right pre-scapular lymph node was slightly enlarged and dark brown. However, histopathologic examination revealed only large amounts of hemosiderin, some brown pigment which was negative to the Prussian blue reaction and stained reddish purple by the PAS technique, and fragments of mycelium.

Mycologic Observations. Many inoculations of the microcolonies onto Sabouraud's maltose agar resulted in the growth of pure cultures of a fungus, the colonies of which reached a diameter of over 40 mm. in 48 to 96 hours (Fig. 11). The growth was white as it grew out from all sides of the black microcolonies but quickly became gray to brown, and finally black with a thin, lightly pigmented peripheral border. The reverse side of the colony had concentric rings of alternating light brown and black. The fungus produced many dark brown, fusiform conidiospores with three to four septa. The third cell from the base was larger and darker than the rest and slightly bulged on one side. The end cells were almost hyaline. Most conidiospores tended to curve slightly on their long axes (Fig. 12). This organism grew slowly without conidiophore formation on ox-blood-agar and failed to grow on Sabouraud's maltose which contained 0.5 mg. of actidione per ml. Figure 13 shows hyphal swelling and early chlamydospore formation under adverse conditions, which in this case were due to growth at 38° to 40° F. Dr. Emory G. Simmons* and Dr. Lucille K. Georg† concurred in identifying this organism as a member of the genus *Curvularia* and Dr. Simmons identified the species as *Curvularia geniculata* (Tracy and Earle) Boedijn.

Case 2

In a dog submitted to the Veterinary Clinic of Texas A. & M. College in May, 1946, a tumefaction in the skin of the foot was diagnosed by histopathologic examination as a mycetoma. The tissue contained numerous dark brown to black granules which were visible grossly. The histopathologic examination revealed many microcolonies

* Dr. Emory G. Simmons, Mycologist (Taxonomy), Pioneering Research Division, Headquarters Quartermaster Research and Development Command, Quartermaster Research and Development Center, U.S. Army, Natick, Mass.

† Dr. Lucille K. Georg, Mycologist, Communicable Disease Center, Laboratory Branch, U.S. Public Health Service, Chamblee, Ga.

of dark brown fungus containing a central core of pigmented, septate hyphae forming occasional swellings and surrounded by a corona of large, dark, thick-walled chlamydospores. The larger colonies were surrounded by granulomatous tissue and fibrous septa similar to those in case 1. Some of these colonies were present in areas previously occupied by the coiled glands of the foot, and the septa were, for the most part, those which normally divide these glands into lobules. Occasionally more than one microcolony was found within an elongated area circumscribed by one fibrous septum. A few very small and apparently early colonies could be found within glandular areas (Fig. 8), and the cells surrounding these colonies varied from epithelioid to true epithelium, many with vacuolated cytoplasm. Also many vacuolated cells were present in the tissue surrounding these colonies. No cultures were made on this case.

Case 3

Case 3 is represented by tissue removed for biopsy from a tumor in the region of the coronary band of a horse at the Veterinary Clinic, Texas A. & M. College, in November, 1939. Grossly, the tissue was fibrous and ulcerated. Many dark brown granules, 0.5 to 1.5 mm. in diameter, were visible on the cut surface. Histopathologic examination revealed many microcolonies of dark brown fungus with a central core of hyphae and an incomplete corona of large, dark brown chlamydospores which formed horseshoe shapes and scroll formations. A few colonies without hyphae formed curved or linear structures (Fig. 7). These microcolonies were surrounded by zones of neutrophils of variable thickness, some several hundred microns wide and others containing only a few neutrophils. Those foci with only a few neutrophils were surrounded by wide zones of macrophages including an occasional multinucleated giant cell, a few plasma cells, and lymphocytes. The tissue surrounding these granulomas was composed of immature fibrous connective tissue with numerous capillaries. None of the discrete septa of mature fibrous connective tissue as seen in cases 1 and 2 was found. Occasional small fragments of pigmented fungus were present in multinucleated giant cells outside of the main colonies. The ulcerated surface had only a few fragments of epithelium and no accessory skin structures were found.

Case 4

Case 4 differs from the other cases reported here in that the lesions were present in the abdominal cavity instead of on the terminal portion of an appendage. The animal, an adult female dog, was submitted for clinical examination because of distention of the abdomen

which had been noticeable for approximately 3 weeks. An exploratory celiotomy revealed adhesions between the abdominal viscera. A piece of small intestine, a portion of kidney, and a piece of granulomatous tissue containing numerous small necrotic foci, all fixed in formalin, were submitted to this laboratory for examination.*

Histopathologic study revealed the granulomatous tissue to be composed of many small inflammatory foci surrounded by mature fibrous connective tissue. The centers of the foci contained one to several microcolonies (grains) of the fungus with a core of hyphae which measured 2 to 3 μ in diameter and a periphery of hyphae and hyphal swellings or chlamydospores (Fig. 9). The chlamydospores measured approximately 7 to 10 μ in diameter. The colonies had no pigmentation; they did not stain with Giemsa's or hematoxylin and eosin stains, and only occasional hyphae were stained by Gram's method. The number of chlamydospores in these colonies was relatively small when compared to the number occurring in the other three cases. Surrounding the colonies were zones of neutrophils which were in turn surrounded by epithelioid cells and fibrous connective tissue (Fig 5). Many of the epithelioid cells contained fine particles which stained reddish purple by the PAS technique.

This case represents the white-grained maduromycosis. Cultures could not be obtained because all of the specimen was received in formalin.

DISCUSSION

The fact that only four cases of maduromycotic mycetomas have been diagnosed among many thousands of biopsies and necropsies suggests that this lesion will not be seen often in the average veterinary practice. However, the importance of such lesions to a veterinary practitioner is magnified by the probability that they can and often do persist for years in spite of therapy. Most of the cases mentioned here have been found in the southern regions of the United States, thus suggesting that maduromycosis, in animals as in man, is primarily a subtropical or tropical disease. The fungi causing maduromycosis in the human are primarily saprophytes in the soil or on plants. The one etiologic agent identified here, *Curvularia geniculata*, is saprophytic and occasionally parasitic on plants such as native grasses and cereals as well as cabbage, flax, and pea seed.²³ No report has been found which mentioned fungi of this genus as affecting animal or man. The genus *Curvularia* is closely related taxonomically to the genus *Helminthosporium* which Georg¹⁰ identified from what apparently is an unreported case of maduromycotic mycetoma in a dog.

* Pathologic specimen submitted by Dr. N. W. Porter, Denison, Texas.

The fungi appearing in another dog (case 2) and in the foot of the horse (case 3) were very similar in structure and pigmentation to *Curvularia geniculata* as it appeared in case 1. The fungus in case 4 was quite different and certainly is not related to any of those found in the other cases. There was nothing in the available information on case 4 to explain the presence of the fungus in the abdominal cavity.

Careful study of serial sections of these lesions gives a definite impression that the infection of the dogs' feet arose in the glandular structures. This is supported by the observation of small colonies in glandular areas and the presence of the original trabeculae surrounding the glands as well as the granulomatous nodules (Fig. 8). The occurrence of infections in the highly glandular dermis of the interdigital spaces of three feet of one dog does not suggest trauma or penetrating wounds as the basic element in the establishment of the infections.

Since many of these fungi, especially *Curvularia*, are inhibited by actidione, this material should be avoided in attempts to isolate them. The persistence of the lesion over long periods of time, even years, should make it possible to get repeated cultures and thus to confirm the identity of the etiologic agent.

SUMMARY

The literature on maduromycotic mycetomas in man and similar lesions in animals is briefly reviewed and discussed.

Three cases of maduromycotic mycetomas are reported in dogs and one case in a horse. Three of the fungi were of the black-grained type and one of the white-grained type.

Curvularia geniculata is identified as an etiologic agent in multiple maduromycotic mycetomas affecting the feet of a dog.

REFERENCES

1. Gammel, J. A. The etiology of maduromycosis with a mycologic report on two new species observed in the United States. *Arch. Dermat. & Syph.*, 1927, 15, 241-284.
2. Gill. Cited by Castellani and Chalmers.⁹
3. Colebrook. Cited by Castellani and Chalmers.⁹
4. Godfrey. Cited by Castellani and Chalmers.⁹
5. Vandyke Carter. Cited by Castellani and Chalmers.⁹
6. Boyce and Surveyor. Cited by Castellani and Chalmers.⁹
7. Pinoy, E. Actinomycoses et mycétomes. *Bull. Inst. Pasteur*, 1913, 11, 929-938 and 977-984.
8. Chalmers, A. J., and Archibald, R. G. A Sudanese maduromycosis. *Ann. Trop. Med.*, 1916-17, 10, 169-222.
9. Castellani, A., and Chalmers, A. J. *Manual of Tropical Medicine*. William Wood & Co., New York, 1919, ed. 3, pp. 2110-2130.

10. Conant, N. F.; Smith, D. T.; Baker, R. D.; Callaway, J. L., and Martin, D. S. Manual of Clinical Mycology. W. B. Saunders Co., Philadelphia, 1954, ed. 2, pp. 240-253.
11. Brumpt, E. Précis de Parasitologie. Masson et Cie, Paris, 1949, ed. 6, 2, 1680-1683.
12. Puestow, K. L. Maduromycosis. A contribution to the study of maduromycosis, with report of a case of infection with *Aspergillus nidulans*. *Arch. Dermat. & Syph.*, 1929, 20, 642-664.
13. Aronson, S. M.; Benham, R., and Wolf, A. Maduromycosis of the central nervous system. *J. Neuropath. & Exper. Neurol.*, 1953, 12, 158-168.
14. Zimmerman, L. E. Fatal fungus infections complicating other diseases. *Am. J. Clin. Path.*, 1955, 25, 46-65.
15. Gleiser, C. A. Mucormycosis in animals. A report of three cases. *J. Am. Vet. M. A.*, 1953, 123, 441-445.
16. Robinson, V. B. Third Annual Seminar of the American College of Veterinary Pathologists, 1952, Case number 12.
17. Seibold, H. R. Mycetoma in a dog. *J. Am. Vet. M. A.*, 1955, 127, 444-445.
18. Riser, W. H. In: Canine Medicine, Hoskins, H. P.; Lacroix, J. V., and Mayer, K. (eds.). American Veterinary Publications, Inc., Evanston, Ill., 1953, pp. 447-448.
19. Georg, L. K. Personal communication, 1955.
20. Akün, R. Eine chromoblastomykosisähnliche Pilzkrankheit beim Pferde. *Zentralbl. f. allg. Path. u. path. Anat.*, 1953, 90, 294-297.
21. Symmers, D., and Sporer, A. Maduromycosis of the hand with special reference to heretofore undescribed foreign body granulomas formed around disintegrated chlamydospores. *Arch. Path.*, 1944, 37, 309-318.
22. Lillie, R. D. Histopathologic Technic and Practical Histochemistry. The Blakiston Co., Inc., New York, 1954, p. 335.
23. Sprague, R. Diseases of Cereals and Grasses in North America (Fungi, Except Smuts and Rusts). Ronald Press Co., New York, 1950, pp. 324-326.

LEGENDS FOR FIGURES

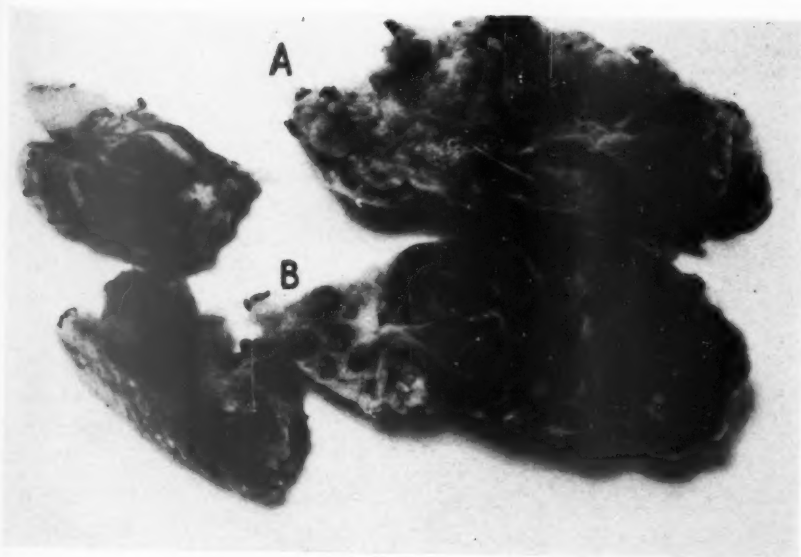
- FIG. 1. Case 1. Right front foot of dog with maduromycotic mycetoma, showing over-all enlargement with fistulous openings. $\times 1$.
- FIG. 2. Case 1. Left rear foot of dog with maduromycotic mycetoma, showing tumefaction between toes and pigmentation of the skin. $\times 1$.
- FIG. 3. Case 1. Granulation tissue of maduromycotic mycetoma with pigmented microcolonies (grains) from left rear foot (Fig. 2). A. Outside of dissected mass with embedded microcolonies. B. Cut surface of dissected mass shows small granulomatous foci with trabeculae. Black specks in background are loose microcolonies. $\times 1\frac{1}{2}$.



1



2



3

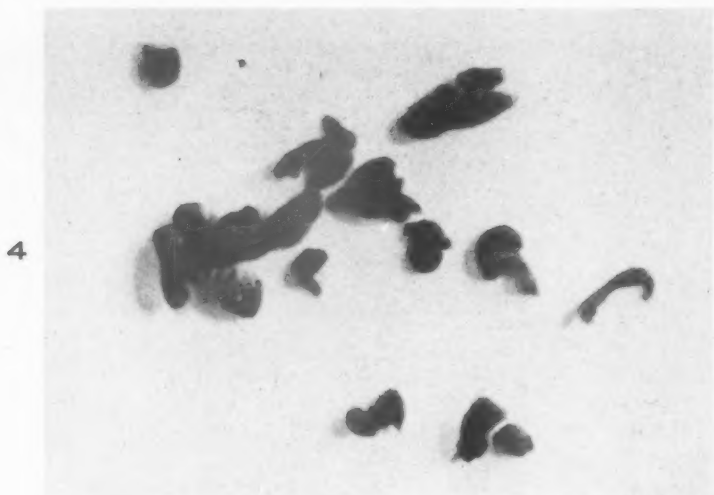
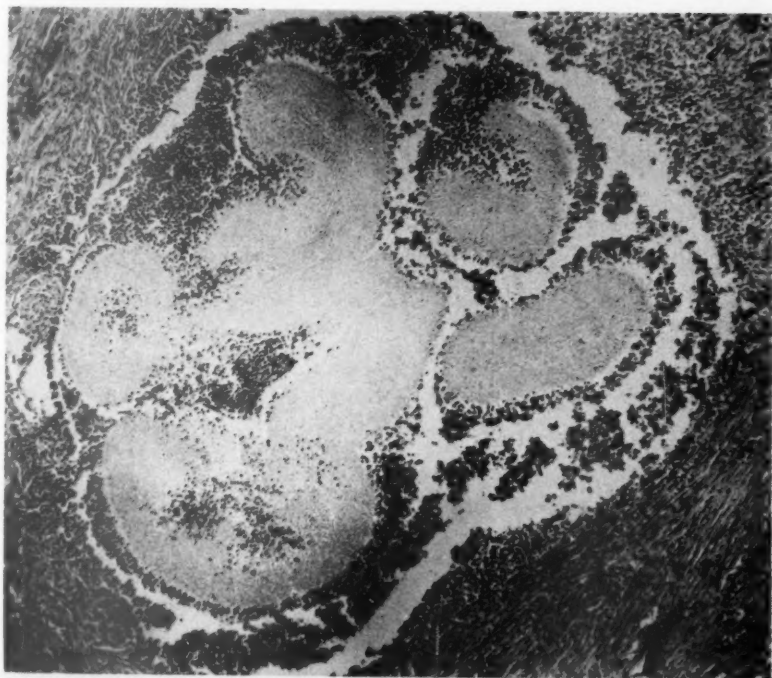


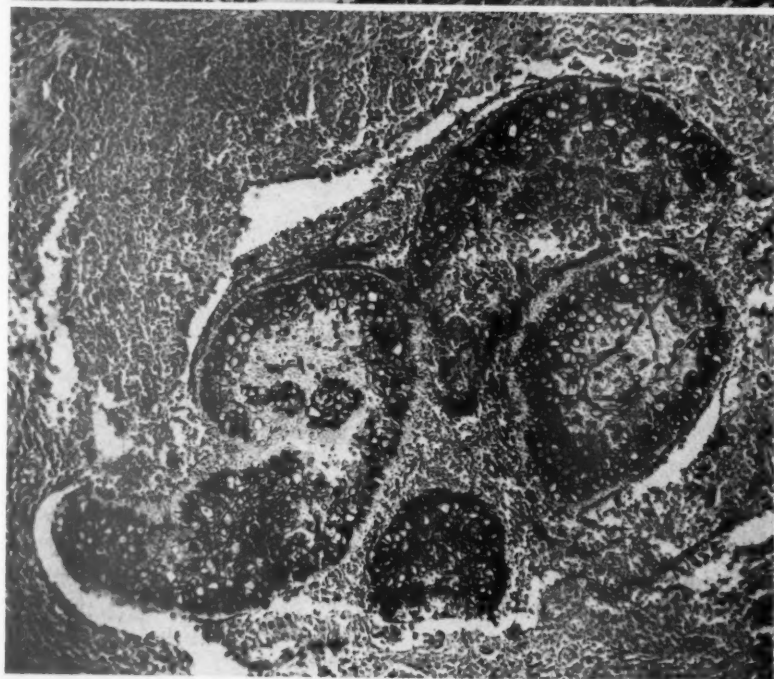
FIG. 4. Case 1. Microcolonies or grains from fistulas of right front foot (Fig. 1).
× 12.

FIG. 5. Case 4, dog. Several microcolonies of unpigmented fungus surrounded by purulent exudate. Hematoxylin and eosin stain. × 70.

FIG. 6. Case 1, dog. Several microcolonies of pigmented fungus (*Curvularia geniculata*) surrounded by purulent exudate and granulation tissue. Pigment gives detail to the colonies. These may be compared with unpigmented colonies in Figure 5 which lack visible detail. Hematoxylin and eosin stain. × 70.



5



6

7

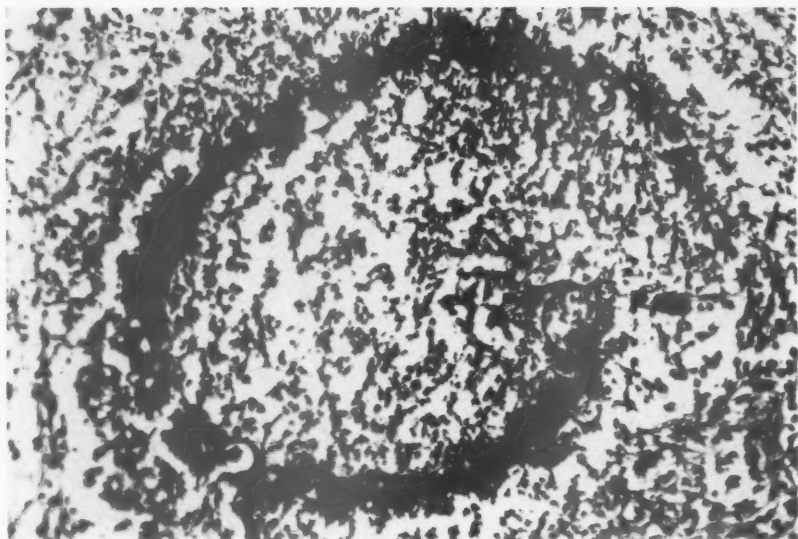
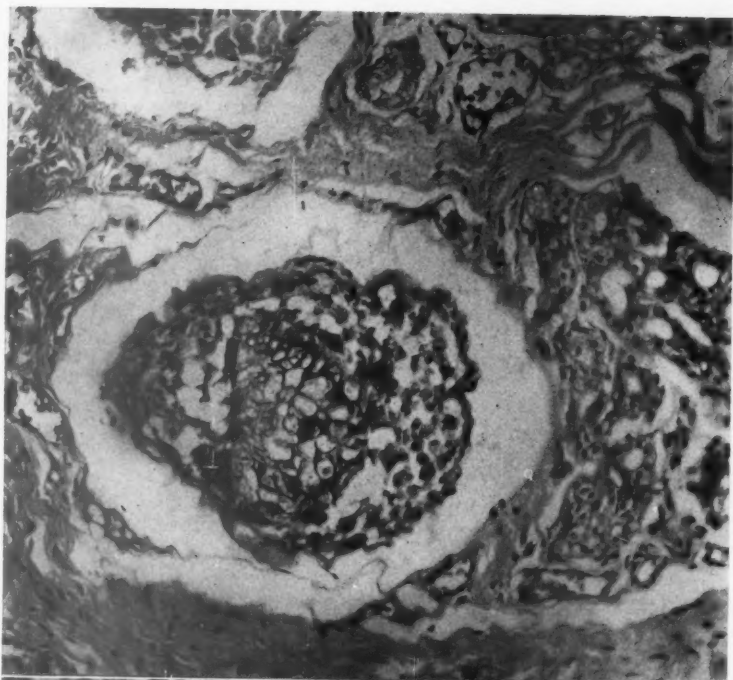


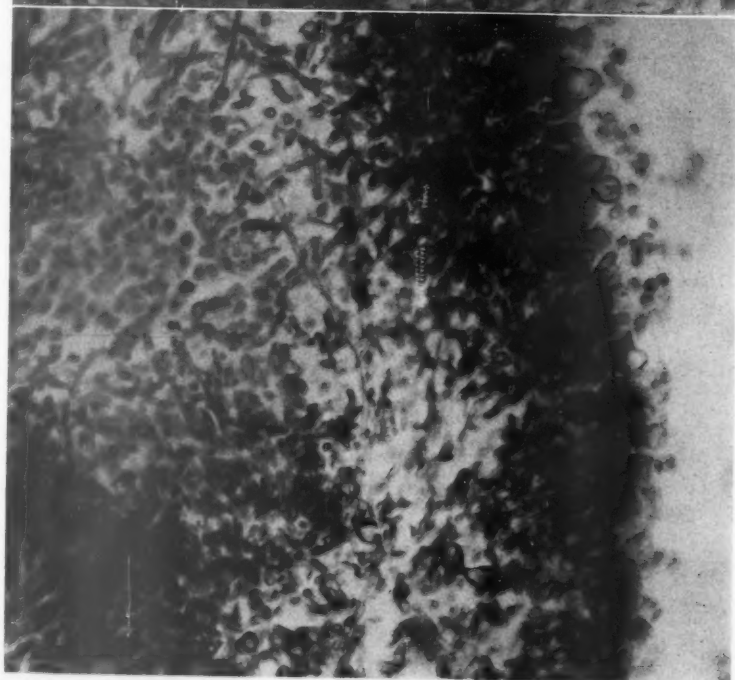
FIG. 7. Case 3, horse. Microcolony without hyphae in focus of purulent exudate. Only chlamydospores compose the colony. Other colonies of this case have hyphae and resembled those of Figure 6. Hematoxylin and eosin stain. $\times 80$.

FIG. 8. Case 2, dog. Pigmented microcolony composed of chlamydospores in glandular area of the subcutis of dog's foot. Inflammatory cells are minimal. Preformed trabeculae may be noted. Hematoxylin and eosin stain. $\times 200$.

FIG. 9. Case 4, dog. Portion of unpigmented microcolony of fungus showing dense peripheral zones of hyphae and chlamydospores and a central core of hyphae. For comparison with Figure 10 showing pigmented fungus. Gridley's fungus stain. $\times 450$.



8



9

10

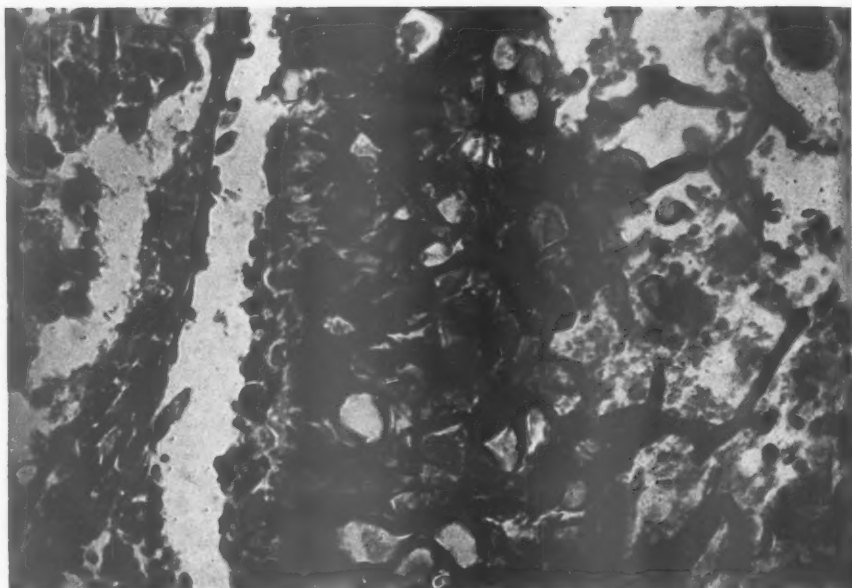
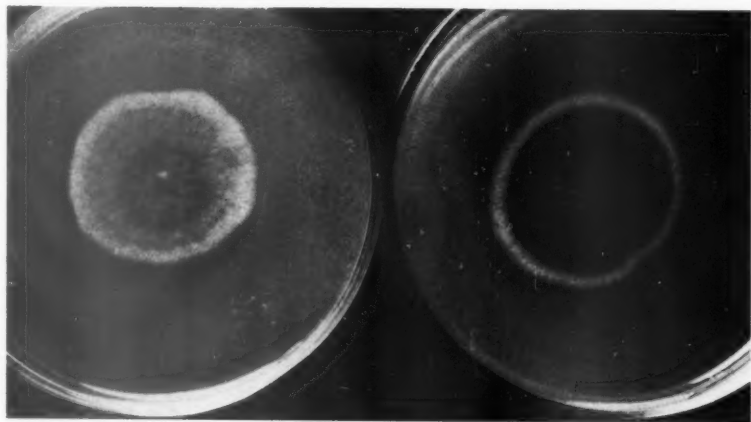


FIG. 10. Case 1. Portion of pigmented microcolony of fungus (*Curvularia geniculata*) showing peripheral zone of hyphae and chlamydospores and central zone of hyphae. Size of hyphae and chlamydospores of the organism shown in Figure 9 (case 4) may be compared with those of this organism. Hematoxylin and eosin stain. $\times 450$.

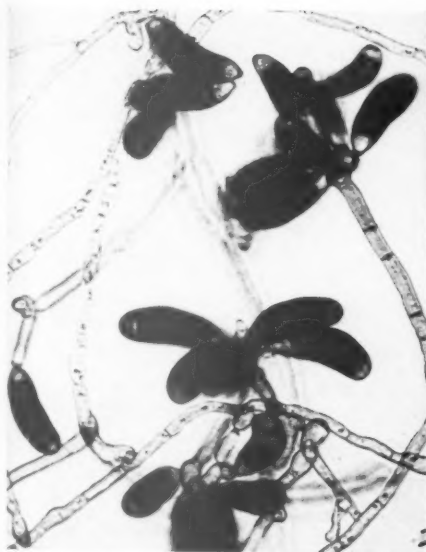
FIG. 11. Two 96-hour-old colonies of *Curvularia geniculata* isolated from case 1. The dark colony contains many pigmented conidiospores (Fig. 12); the light colony contains relatively few pigmented conidiospores. $\times 34$.

FIG. 12. Pigmented, curved conidiospores of *Curvularia geniculata* from case 1. Slide culture on Sabouraud's maltose agar. $\times 540$.

FIG. 13. Case 1. Hyphal swellings and early chlamydospore formation by *Curvularia geniculata* grown under adverse conditions (Refrigeration at 40° F.). $\times 500$.



11



12



13

AUTO-IMMUNE HEMOLYTIC ANEMIA

II. MORPHOLOGIC OBSERVATIONS AND CLINICOPATHOLOGIC CORRELATIONS*

HENRY RAFFAPORT, M.D., and WILLIAM H. CROSBY, M.D.

From the Armed Forces Institute of Pathology, Washington, D.C., the Department of Pathology, Mount Sinai Hospital, Chicago, Ill., and the Department of Hematology, Walter Reed Army Institute of Research, Washington, D.C.

Current knowledge of the morphologic changes in the spleen and other organs in acquired hemolytic anemia is based on observations in single cases or small series,¹⁻¹⁶ and little, if any, attempt has been made to correlate clinical and hematologic findings with the histologic appearance of the spleen. The present study was undertaken to ascertain the pathologic changes in the spleen and other organs in a large number of cases of auto-immune hemolytic anemia (AHA) and to attempt a correlation of these changes with certain clinical and laboratory findings. The hematologic observations on approximately the same group of cases were evaluated previously with reference to their prognostic implications.¹⁷

MATERIAL AND METHODS

Fifty cases of acquired hemolytic anemia from the files of the Armed Forces Institute of Pathology were selected for this study. All cases included in the series satisfied two requirements: (1) Tissue from the spleen was available for microscopic examination, and (2) the diagnosis of acquired hemolytic anemia of the auto-immune type had been established clinically beyond reasonable doubt. The cases were contributed to the Armed Forces Institute of Pathology from many sources and the completeness of the records and the amount and quality of pathologic material vary with the contributors. Data dealing with the gross features of the spleen and other organs were collected from surgical and necropsy reports; only the information concerning microscopic features was derived from personal observation. Of the 50 spleens examined, 41 had been removed surgically and nine were obtained at necropsy. Ten of the spleens removed surgically and four of those obtained post mortem were from patients in whom AHA was associated with malignant lymphoma. Lymph nodes were studied in cases from which they had been re-

* Received for publication, June 20, 1956.

Presented, in part, at the Sixth Congress of the International Society of Hematology, Boston, August 27 to September 1, 1956.

moved for diagnostic purposes or in conjunction with splenectomy, or at necropsy. With the exception of hepatic biopsies of seven cases and marrow biopsies of two, other tissues represented specimens derived from necropsies of 24 cases.

Sections from spleens and other organs were stained with hematoxylin and eosin, Masson's trichrome stain (Mallory's modification), and Verhoeff's stain for elastic tissue. Reticulum was demonstrated by the methods of Gridley¹⁸ and Snook¹⁹ and by the periodic acid-Schiff (PAS) reaction. The Prussian blue reaction for iron was done according to the method of Gomori.²⁰

In all fatal cases the clinical records, necropsy protocols, and histologic sections were reviewed in an attempt to establish the immediate and contributory causes of death. An effort was made to trace all living patients and to obtain recent clinical and hematologic follow-up data from which to judge the success of splenectomy.¹⁷

The spleens from 28 patients with hereditary spherocytosis and from three with familial non-spherocytic hemolytic disease, previously reported,²¹ were studied for comparison.

GROSS AND MICROSCOPIC OBSERVATIONS

Spleen

The average weight of the spleen in 13 patients with malignant lymphoma was 1,427 gm. (range, 250 to 4,800 gm.); in seven the spleen weighed more than 1,000 gm. In 36 patients without malignant lymphoma the average weight of the spleen was 650 gm. (range, 148 to 1,670 gm.); in only five did its weight exceed 1,000 gm. In comparison, the average weight of the spleen in 28 unselected cases of hereditary spherocytosis was 870 gm. (range, 430 to 1,410 gm.).

In general, the spleen had a smooth capsule, which was thickened in about half the cases. Splenic consistency was normal in approximately 50 per cent of the cases. In half of the remaining cases the spleen was firm and in the other half, soft. Scraping usually yielded little or no pulp, but in some instances considerable amounts of blood. Splenic infarcts were found in 20 per cent of patients with malignant lymphoma and in 26 per cent of patients without malignant lymphoma.

The histologic appearance of the spleens of patients with AHA was not uniform. The variations encountered resulted primarily from the relative degrees of congestion of pulp cords and sinuses, and the presence or absence of extramedullary hematopoiesis. Splenic involvement by coexistent conditions, such as malignant lymphoma, which occurred in almost one third of our cases, contributed to the diversified pathologic picture.

Architecture. The architecture of the spleen was preserved even

though it was modified by certain features that will be described in detail under separate subheadings. Under low magnification, congestion of either sinuses or splenic cords, or both, was impressive, and, as a consequence of this change, the malpighian corpuscles and also the trabeculae appeared to be more widely spaced than in normal spleens. Otherwise no striking alteration in the architectural pattern was observed unless there was involvement by malignant lymphoma.

Splenic Cords and Sinuses. In 18 spleens the cords were severely congested, in contrast to the sinuses which either were empty or contained only small to moderate numbers of red blood cells (Figs. 1 and 2; Table I). The pathologic picture in these spleens resembled that

TABLE I
Red Blood Cell Content of Splenic Cords and Sinuses in
Relation to Spherocytosis
Group I. Splenic Cords Severely Congested and Red Blood Cell
Content of Sinuses Not Significantly Increased

Accession number	Spherocytosis	Osmotic fragility*	Red blood cell content	
			Cords	Sinuses
314905†	+	Marked increase	++++	0
166580	+	Marked increase	++++	+
211621†‡	+	Marked increase	++++	+
200203	+	Marked increase	++++	+
490583	+	Marked increase	++++	+
500865	+	Marked increase	++++	+
126394‡			++++	++
234089	0	Normal	++++	++
268756	+	Marked increase	++++	++
302465‡	+	Marked increase	++++	++
550856	+	Moderate increase	++++	++
575449	0	Normal	++++	++
495207			++++	+
499152‡	+	Slight increase	+++	+
541813	+	Slight increase	+++	+
543491	0	Normal	+++	+
593162	+	Normal	+++	+
609537	+	Marked increase	+++	0

* Normal = 0.02 or less above normal control.

Slight increase = 0.03 to 0.05 above normal control.

Moderate increase = 0.06 to 0.08 above normal control.

Marked increase = 0.09 or more above normal control.

† Patients with malignant lymphoma.

‡ Spleens obtained at necropsy.

of hereditary spherocytosis. The sinuses in some instances were compressed as a result of widening of the heavily congested splenic cords, but this feature was rarely as pronounced as in hereditary spherocytosis. In 21 spleens congestion was equally prominent in cords and sinuses or the difference was slight (Fig. 3; Table II). In a third group comprising 11 patients, the splenic cords were narrow and contained relatively few red blood cells while the sinuses usually were congested (Fig. 4; Table III).

TABLE II
*Red Blood Cell Content of Splenic Cords and Sinuses in
 Relation to Spherocytosis*
*Group II. Congestion of Cords and Sinuses Either
 Equally Pronounced or Only Slightly Different*

Accession number	Spherocytosis	Osmotic fragility*	Red blood cell content	
			Cords	Sinuses
285665	+	Normal	++++	++++
549129	+		++++	++++
195298†	O	Normal	++++	++++
287449	+	Normal	++++	++++
307140†	+		++++	++++
331560	+	Slight increase	++++	++++
486196	+	Slight increase	++++	++++
501912†	+	Slight increase	++++	++++
626988	O	Normal	++++	++++
106980	O	Normal	++++	++++
192869	O	Normal	++++	++++
497314†	O	Normal	++++	++++
311839	O	Normal	++++	++++
483287†	O	Normal	++++	++++
544313			++++	++++
215470	+	Moderate increase	++++	++
557297	O		++++	++
569024†	O	Normal	++++	++
125000†	O	Normal	++	++++
127761†	O	Normal	++	++++
513991†	O	Normal	++	++

* Normal = 0.02 or less above normal control.

Slight increase = 0.03 to 0.05 above normal control.

Moderate increase = 0.06 to 0.08 above normal control.

† Patients with malignant lymphoma.

‡ Splenic obtained at necropsy.

TABLE III
*Red Blood Cell Content of Splenic Cords and Sinuses in
 Relation to Spherocytosis*
*Group III. Sinuses Moderately to Severely Congested and
 Cords Containing Relatively Few Red Blood Cells*

Accession number	Spherocytosis	Osmotic fragility*	Red blood cell content	
			Cords	Sinuses
99031†	O	Normal	+	++++
261543†	O	Normal	+	++++
275088	O	Normal	+	++++
300853†		Normal	+	++++
523966	O	Normal	+	++++
335892†	O	Normal	O	++++
582058	O	Moderate increase	O	++++
594007	O	Normal	O	++
626829	O	Normal	O	++
172923	O	Normal	+	++
327642†	O	Normal	+	++

* Normal = 0.02 or less above normal control.

Moderate increase = 0.06 to 0.08 above normal control.

† Patients with malignant lymphoma.

TABLE IV
Osmotic Fragility and Spherocytosis in Relation to Congestion of Splenic Cords and Sinuses

Group	Red blood cell content		Number of cases	Spherocytosis			Osmotic fragility ^a					
							Increase					
							Marked	Moderate	Slight	Total	Normal	Undetermined
I (Table I)	++++ to +++++	0 to ++	18	% 72	% 17	% 11	% 50	% 6	% 11	% 67	% 22	% 11
II (Table II)	++ to +++++	++ to +++++	21	38	57	5		5	14	19	67	14
III (Table III)	0 to +	++ to +++++	11		91	9		9		9	91	
Total with data			50									

* Normal = 0.02 or less above normal control.

Slight increase = 0.03 to 0.05 above normal control.

Moderate increase = 0.06 to 0.08 above normal control.

Marked increase = 0.09 or more above normal control.

The degree of congestion of splenic cords and sinuses, respectively, was graded from 1 plus to 4 plus, and correlated with the osmotic fragility and the presence of spherocytosis (Tables I to III). The results, summarized in Table IV, indicate a high degree of correlation between spherocytosis and increased osmotic fragility on the one hand and the degree of congestion of the splenic cords on the other.

In 12 spleens there was hyperplasia of the cellular reticulum of the splenic cords accompanied by an increase in the number of lymphocytes and macrophages; most of the latter contained hemosiderin. Plasma cells were present in increased numbers in seven cases and polymorphonuclear leukocytes in nine. Eosinophils were not prominent. In 13 spleens the reticulum cells within the splenic cords were widely scattered, particularly when congestion of the cords was pronounced. It is possible that in some instances hyperplasia of the cellular reticulum may have been masked by the congestion.

Prominence of the lining cells of the splenic sinuses and particularly of their nuclei, a constant feature in hereditary spherocytosis (Fig. 5), was observed in approximately one half the cases of AHA. Meulengracht²² pointed out that normally the sinuses are lined by "endothelial" cells (rod cells) measuring as much as 100 μ in length (Fig. 9); thus, in cross section, nuclei are few in relation to cytoplasmic processes (Figs. 2 and 10). In contrast, the splenic sinuses of patients with chronic hereditary hemolytic anemia are lined with closely set, large, round, prominent nuclei surrounded by minimal amounts of cytoplasm. Meulengracht indicated that this "adenomatoid" appearance of the sinuses (Fig. 5) can be explained only by assuming that the individual cells are shorter than normal. We find this explanation plausible, for the shorter the lining cell, the greater the likelihood that its nucleus will be included in a cross section of a sinus. Because a greater number of such shortened lining cells would be needed to cover a given surface area, the numerical increase of nuclei on cross section of a sinus is indicative of true hyperplasia of the lining cells. In AHA prominence and hyperplasia of cells lining the sinuses were frequently, but not uniformly, associated with extreme congestion of the splenic cords. In sections stained by the Prussian blue method, the lining cells, which are macrophages rather than endothelial cells, invariably contained iron-positive material, either as coarse granules or as a diffuse impregnation of the cytoplasm.

The lumina of the sinuses varied from almost complete emptiness to extreme engorgement. In most spleens the red blood cells appeared to be evenly distributed throughout the lumina, but there was a tendency toward crowding at the center in some. Clumps of red blood cells, strongly suggestive of intravascular agglutination, were observed in two cases, in one of which the auto-agglutinin titer was very high. Often the sinuses contained numerous pigment-laden macrophages. In many areas these appeared to represent lining cells that had been discharged into the lumen. This, however, was difficult to prove; once macrophages are within the lumina of the sinuses their origin is impossible to ascertain. This point is illustrated in Figure 10 in which a macrophage is shown penetrating the sinus wall, presumably on its way into the lumen. After completion of its passage through the wall of the sinus, this cell might have been interpreted as a lining cell.

Erythrophagocytosis (Fig. 11) was observed in 40 (80 per cent) of the spleens of patients with AHA. In the sinuses the cellular outlines of erythrocyte-containing macrophages could be easily recog-

nized, but in the splenic cords red blood cells and macrophages were so crowded together that one could rarely be certain whether the former were extracellular or intracellular. The same difficulty was encountered in spleens removed from patients with hereditary spherocytosis, which may be the reason why some observers²³ have stressed the infrequency of erythrophagocytosis in that disease. As Klemperer²⁴ pointed out, erythrophagocytosis may be masked by the heavy congestion of the cords.

Extramedullary hematopoiesis was found in ten spleens removed surgically and in five spleens obtained post mortem. In most cases, cells of the erythrocytic series predominated to such an extent (Fig. 12) that differentiation from myeloid metaplasia occurring in the myeloproliferative diseases or space-occupying disorders of the bone marrow could be readily accomplished. Usually only a few megakaryocytes and immature granulocytes were observed.

White Pulp. The malpighian corpuscles usually were widely separated. In the cases not associated with malignant lymphoma, they appeared large in seven, small in five, and about normal size in the remainder. Reaction centers were observed in a majority of the spleens. They were large and hyperactive in seven. The outer marginal zone was prominent and widened in 12 cases, distinct but not enlarged in five, inconspicuous or absent in 19. Hyaline deposits,^{2,4} positive with the PAS stain, were seen within some of the reaction centers in 14 cases. Lymphomatous involvement of the spleen was demonstrated in all but one of the cases in which the hemolytic anemia was known to be associated with malignant lymphoma.

Capsule and Trabeculae. No significant alterations were seen in the capsule and trabeculae, which usually were of normal thickness. In only six instances was the capsule, the trabeculae, or both, slightly to moderately thickened.

Blood Vessels. Vascular changes were not prominent. Venous thrombosis was found in two cases. However, infarcts were relatively common, occurring in 24 per cent of all cases. Homogeneous, acidophilic, subintimal deposits, which gave positive reactions for fibrin and with PAS stains but showed marked tinctorial variations with connective tissue stains, were observed in ten spleens, in three of which malignant lymphoma also was present. This vascular alteration apparently is non-specific.^{25,26} Reduplication of the elastica of the arteries occurred in four cases; the respective ages of these patients were 26, 26, 39, and 55 years.

Deposition of Iron-Containing Pigments. Hemosiderin deposition

was abundant in properly fixed spleens, but in a significant number its extent could not be assessed because of large accumulations of formalin pigment resulting from fixation or prolonged storage in an acid fixative. Failure to preserve tissues in neutral fixatives may lead not only to blurring of cytologic detail but also to false localization,²⁰ presumably because of diffusion of iron-positive material. Nuclei may become iron-positive, and reticulum and connective tissue fibers have such a strong affinity for the ferric ion that they will adsorb it even from extremely dilute solutions.²⁰ Wherever localization of iron-containing pigment could be properly evaluated, however, it was most abundant in the macrophages of the splenic cords. Considerable amounts of hemosiderin were present also in macrophages within the lumina of the sinuses, while only moderate amounts were noted in the sinusoidal lining cells. This difference in hemosiderin content suggests that lining cells which have accumulated large amounts of iron-containing pigment are shed into the lumina of the sinuses which may represent a mechanism of removal of iron from the spleen. The follicles were either devoid of iron-containing pigment (Fig. 13) or exhibited only scattered macrophages with pigment granules. The trabeculae contained varying amounts of iron, and irregularly arranged reticulum fibers were frequently impregnated with iron-containing pigments (Fig. 14). The diffuse impregnation of the fibrillar reticulum, which permitted iron stains to duplicate the pattern usually obtained only in a reticulum stain, is probably an artifact resulting from faulty fixation. Hemosiderotic nodules (Gamna-Gandy bodies) occurred in five spleens but were associated with gross infarcts in only one.

Reticulum and Connective Tissue. An increase in the fibrillar reticulum was observed in 16 cases, being marked in one and slight to moderate in 15. In these spleens, the thick, irregularly arranged argyrophilic fibers were readily differentiated from the delicate fibrillar reticulum that normally exists. Increased fibrillar reticulum was particularly pronounced in spleens in which hemosiderosis was most marked. Occasionally it was associated with focal areas of fibrosis, but in most instances fibrosis was either absent or very slight.

Lymph Nodes

The material consisted of (1) lymph nodes removed for biopsy in cases of malignant lymphoma, in some instances before, in others, after, a diagnosis of AHA was suspected or established; (2) abdominal lymph nodes removed during splenectomy; (3) lymph nodes obtained at necropsy. The most significant abnormalities other than malignant lymphoma in 14 cases were hemosiderosis and erythro-

phagocytosis. The degree of hemosiderosis ranged from minimal to excessive, depending on the stage of the disease at which the lymph nodes were removed. Where sequential biopsies had been performed, hemosiderosis was most marked in the later ones, but the blood transfusions that most of these patients had received may have contributed to its severity. It is worthy of note, however, that hemosiderosis was observed in lymph nodes removed from several patients who had not received transfusions. Erythrophagocytosis was evident also in a considerable number of lymph nodes. Both hemosiderosis and erythrophagocytosis were most pronounced in the medullary and peripheral sinuses and often were confined to these areas. Frequently the sinuses were widely distended with pigment-laden macrophages. In several instances, both hemosiderin and intact erythrocytes could be found within a single macrophage. In those cases in which hemosiderosis was most intense, pigment-laden macrophages were scattered diffusely throughout the pulp of the lymph nodes, and the reticular framework of the sinuses was often impregnated with iron-positive material. Whenever both splenic and superficial lymph nodes from the same patient were examined, erythrophagocytosis and hemosiderosis were considerably more severe in the splenic nodes. Erythrophagocytosis or hemosiderosis, or both, were pronounced in some lymph nodes removed from patients with malignant lymphoma before a diagnosis of AHA was made. In three patients with malignant lymphoma, sections of lymph nodes showed an abundance of PAS-positive, intracellular protein which resembled Russell bodies. It has been suggested that these bodies might be the morphologic manifestation of synthesis of abnormal proteins in neoplastic cells, and that such a synthesis might be a part of the immunologic mechanism responsible for the development of AHA in association with malignant lymphoma.²⁷ Extramedullary hematopoiesis of the lymph nodes was observed in only two instances; both necropsies.

Bone Marrow

Sections of bone marrow were available in 20 instances, and all but two represented necropsy material. Erythroid hyperplasia was pronounced in ten of 13 patients with idiopathic AHA (Fig. 15). In the other three, all of whom had neutropenia and thrombocytopenia, the bone marrow was hypocellular. In one of these, megakaryocytes were absent, granulopoiesis was slight, and erythropoiesis was prominent against the background of an otherwise hypocellular marrow (Fig. 16). A relatively small number of megakaryocytes provided the only variation from a similar picture in the second

patient. In the third, focal areas of depletion of cells alternated with areas of normal cellularity. Of seven patients in whom AHA was associated with malignant lymphoma, erythroid hyperplasia was pronounced in one, moderate in two, slight in one, and absent in three whose bone marrow was extensively infiltrated by malignant lymphoma. The number of megakaryocytes was definitely increased in five patients, three of whom had idiopathic AHA with thrombocytopenia (platelet counts were 15,000, 32,000, 13,500, respectively), while in the remaining two, platelet counts were not available. Erythrophagocytosis was demonstrated in most cases in which the sections of bone marrow were of adequate quality for evaluation of this feature. Hemosiderosis of the bone marrow was always present.

Liver

Sections of liver taken for biopsy were studied in seven patients. The most striking feature was marked hemosiderosis, which was always present in both parenchymal cells and Kupffer cells. Hemosiderosis was equally intense in cells of both types in four instances, in two it was more pronounced in the Kupffer cells, and in one, in the parenchymal cells. Hemosiderosis was even more extensive in sections of liver obtained at necropsy. In most cases, storage of iron-positive pigment had occurred to an equal degree in Kupffer cells and parenchymal cells, but in some it was more pronounced in the parenchymal cells and in others, in the Kupffer cells. In not a single instance was hemosiderosis associated with portal fibrosis.

Another prominent feature in post-mortem sections of the liver was central necrosis of the hepatic lobules. It was observed in seven instances, always in association with severe congestion. The patients in whom such lesions were demonstrated had died with severe anemia (average erythrocyte count, 1.1 million; range, 0.7 to 1.4 million), with the exception of one who also had rheumatic heart disease and whose red blood cell count near the time of death was 2.7 million. Most of the sections of liver obtained at necropsy showed passive congestion, which probably accounted for much of the hepatic enlargement in patients without malignant lymphoma (average weight, 2,523 gm.) and contributed to the size of the livers of those with malignant lymphoma (average weight, 3,077 gm.). Lipidic metamorphosis was observed rarely and, when present, was slight. Extramedullary hematopoiesis was evident in only three livers. Cholelithiasis was an incidental finding in two patients at necropsy. It had produced no clinical manifestations or complications.

Other Organs

The heart was usually enlarged. In most instances cardiac hypertrophy was moderate and probably had developed in response to the increased cardiac output incident to the anemia. The average weight of the heart in ten patients with malignant lymphoma was 423 gm. (range, 210 to 550 gm.). In patients without malignant lymphoma the average weight of the heart was 430 gm. (range, 300 to 625 gm.). Hearts from patients with rheumatic or arteriosclerotic heart disease were not included in this computation. Pulmonary infarcts in two patients and pulmonary embolism in one were part of thrombo-embolic disease; thrombosis of pulmonary vessels was recorded in one instance, and embolic renal infarcts secondary to cardiac mural thrombi were observed in one patient.

The only significant microscopic feature in organs other than spleen, lymph nodes, liver, and bone marrow was hemosiderosis. The kidneys showed iron-positive pigment granules within the epithelium of the distal convoluted tubules and the ascending limbs of the loops of Henle. Hemoglobin casts were present in one patient who died during an acute hemolytic crisis. Other organs in which iron-containing pigment was demonstrated in slight to moderate amounts were the adrenal glands, heart, pancreas, skin, and lung.

Most of the pertinent post-mortem findings are listed in Table V in which the immediate and contributory causes of death and the red blood cell counts nearest the time of death are shown. Thirteen of 27 patients died of cardiac failure in the absence of intrinsic cardiac disease. For these patients the average red blood cell count nearest the time of death was 1.17 million, ranging between 0.7 and 1.8. For ten patients who died from causes other than cardiac failure or acute hemorrhage, the comparable average red blood cell count was 3.02 million and the range was 1.6 to 5.0. In only two patients of the former group was the red blood cell count nearest the time of death above 1.5 million, and in only one of the latter group was it below 2.5 million.

DISCUSSION

The principal alterations in the surgical and necropsy material from 50 patients with AHA were found in the spleen except in symptomatic AHA in which evidence of the underlying disease process appeared in other organs also. We were impressed with the great variations encountered, both on gross and on microscopic examination. For instance, the size of the spleen varied considerably and, in the

TABLE V
Causes of Death in Auto-immune Hemolytic Anemia

Acc. no.	Immediate cause of death	Contributory causes of death and associated conditions	Red blood cell count nearest time of death (millions/cmm.)
99031	Myocardial infarct due to atherosclerotic coronary arterial disease with thrombosis	Hodgkin's disease; adenocarcinoma of colon with metastases to regional lymph nodes and liver	3.9
106980	Cardiac failure due to anemia, with central hepatic necrosis; bronchopneumonia (terminal)		0.9
111550	Cardiac failure due to anemia, with pulmonary edema, bilateral hydrothorax, and central hepatic necrosis		1.3
125000	Cardiac failure due to anemia, with pulmonary edema	Non-bacterial thrombotic endocarditis of aortic valve; thrombophlebitis, femoral vein	1.2
126394	Cardiac failure due to anemia, with pulmonary edema	Rheumatic heart disease, inactive, with stenosis and insufficiency of mitral valve	1.0
127761	Cardiac failure due to anemia, with central hepatic necrosis		2.7
132806	Probable transfusion reaction (febrile, non-hemolytic)		2.9
192869	Cardiac failure due to anemia, with pulmonary edema and pulmonary infarct		1.2
195298	Terminal febrile illness, type and cause undetermined	Lymphosarcoma, generalized	3.3
211621	Cardiac failure due to anemia, with pulmonary edema	Acute tracheobronchitis; chronic lymphocytic leukemia	0.9
261543	Cardiac failure due to anemia, with pulmonary edema	Hodgkin's disease; thrombosis of pulmonary vessels	1.7
287449	Unknown; no necropsy		1.0
288784	Cardiac failure due to anemia, with pulmonary edema and central hepatic necrosis		0.8
300853	Unknown; no necropsy	Hodgkin's disease	3.2
302465	Acute renal failure due to lower nephron nephrosis secondary to acute intravascular hemolysis		2.7
307140	Unknown; no necropsy	Chronic lymphocytic leukemia	2.5
311839	Cardiac failure due to anemia, with pulmonary edema and central hepatic necrosis		0.7
314995	Cardiac failure due to anemia, with pulmonary edema and central hepatic necrosis	Pulmonary embolism; chronic lymphocytic leukemia	1.3
327642	Unknown; no necropsy	Malignant lymphoma, lymphocytic type, follicular	2.9
423287	Uremia due to chronic pyelonephritis	Lymphosarcoma	2.3
426196	Acute hepatitis (homologous serum jaundice) secondary to transfusions of pooled plasma at time of splenectomy		5.0
497344	Cardiac failure due to anemia, with pulmonary edema	Myocardial thrombosis of heart	3.4
499152	Cardiac failure due to anemia, with pulmonary edema	Chronic lymphocytic leukemia	1.8
501912	Gastro-intestinal hemorrhage due to thrombocytopenia	Chronic lymphocytic leukemia	1.0

483287	Uremia due to chronic pyelonephritis	5.0
48606	Acute hepatitis (homologous serum jaundice) secondary to transfusion of pooled plasma	5.0
492324	Cardiac failure due to anemia, with pulmonary edema	1.8
49257	Cardiac failure due to anemia, with pulmonary edema	1.0
504912	Gastro-intestinal hemorrhage due to thrombocytopenia	2.0
513991	Terminal bronchopneumonia; malnutrition, severe	1.6
523966	Pylephlebitis with thrombosis of portal veins, multiple hepatic abscesses, and generalized pyemia (<i>Pseudomonas aeruginosa</i>)	2.5
543491	Hemolytic <i>Staphylococcus aureus</i> septicemia secondary to suppurative prostatitis	2.6
550856	Cardiac failure due to anemia and arteriosclerotic heart disease, with pulmonary edema	1.9
557302	Unknown; no necropsy	2.1
560024	Gastro-intestinal hemorrhage due to thrombocytopenia	1.0
575449	Cardiac failure due to anemia, with pulmonary edema, pulmonary infarcts, and central hepatic necrosis (18 hours after splenectomy)	1.4
597533	Unknown; no necropsy	5.0
	Actual thromboid of heart	
	Chronic lymphocytic leukemia	
	Chronic lymphocytic leukemia	
	Reticulum cell sarcoma, generalized	
	Pseudomembranous colitis, postoperative	
	Subacute hepatitis (homologous serum jaundice); thrombotic purpura	
	Chronic lymphocytic leukemia	
	Chronic lymphocytic leukemia; subdural hematoma	
	Uncertain; clinical observations during terminal illness (8 months after splenectomy) suggestive of rheumatic heart disease	

absence of malignant lymphoma, was dependent largely on its blood content.

In hereditary spherocytosis the marked congestion of the splenic cords has been attributed to selective trapping of spherocytes, which, because of their thickness, cannot readily pass through the slit-like spaces (stomas) between the lining cells of the sinuses.^{28,29} The existence of such spaces is well established.³⁰ We could demonstrate them easily in some of the spleens in which the sinuses were dilated (Figs. 9 and 10), but they are difficult to visualize in normal spleens unless the sinuses are distended by perfusion. This is consistent with Björkman's³⁰ calculations that the stomas cannot be visualized when the diameter of the sinuses is 10 μ or less; however, as the diameter of the sinuses increases the stomas gradually become wider. Morphologic and experimental evidence in support of selective trapping of red blood cells in hereditary spherocytosis has been brought forth by a number of workers.³¹⁻³³

Because the histologic appearance of some of the spleens in our cases closely resembled that characteristically seen in hereditary spherocytosis, we tried to determine whether in AHA any correlation existed between spherocytosis or increased osmotic fragility, or both, and congestion of the splenic cords. The results of this study are listed in Tables I to IV. Because spherocytosis may be a transient phenomenon in AHA, ideally the blood smears examined should have been obtained close to the date of splenectomy. Since the

material was contributed from sources over which we had no control, we frequently had to be satisfied with undated blood smears and fragility determinations made during the time the patient was in the hospital for splenectomy, or during his terminal hospital stay, if the spleen was obtained at necropsy. In spite of these drawbacks, our findings suggest that a high degree of positive correlation between spherocytosis and congestion of splenic cords does exist. Moreover, in all patients in whom increase of osmotic fragility was classified as marked (Table I), congestion of the splenic cords was maximal. This suggests that the correlation may be both qualitative and quantitative. Our contention that congestion of splenic cords in AHA is largely dependent upon the presence of spherocytes in significant numbers is further supported by a similar situation existing in the congenital hemolytic anemias. As has been demonstrated previously, sections from spleens of patients with congenital non-spherocytic hemolytic disease show almost complete absence of red blood cells in the cords.²¹ We believe, therefore, that the histologic picture of congestion of splenic cords is not pathognomonic of congenital hemolytic anemia,³⁴ nor is the absence of this picture in hemolytic disease indicative of an acquired form. Congestion of splenic cords, or its absence, merely indicates whether the hemolytic disease is predominantly spherocytic or non-spherocytic.

While congestion of the splenic cords is highly characteristic of spherocytosis, it is not specific. We have observed it in hereditary ovalocytosis (Fig. 7), in the early phase of sickle cell anemia (Fig. 8), in acute splenic enlargement following exposure of patients with sickle cell trait to high altitudes, and following massive transfusions immediately preceding splenectomy or shortly before death. We are not aware of its existence in other conditions. Suggestions that cord congestion may be an artifact related to the surgical procedure or other factors are not well supported. We have not seen it in spleens removed for idiopathic thrombocytopenic purpura uncomplicated by hemolytic anemia, or in normal spleens removed for traumatic laceration of the capsule or in connection with gastrectomy.

In the classical descriptions of the spleens of patients with congenital spherocytic hemolytic anemia,^{22,28,34-39} congestion of the splenic cords usually is associated with relatively empty and sometimes with compressed sinuses. A similar, though frequently less pronounced, contrast between the red blood cell content of splenic cords and that of sinuses was observed in about one third of the spleens of patients with AHA, particularly if the increase in the osmotic fragility of the

erythrocytes was marked, indicating pronounced spherocytosis (Table I). On the other hand, in patients with little or no spherocytosis, in most of whom osmotic fragility was either normal or only slightly increased, the sinuses usually were congested. In a number of instances congestion of sinuses was associated with narrow cords almost completely devoid of red cells (Table III). Our observations indicate that congestion of cords is consistent with a significant degree of spherocytosis; congestion of sinuses, with paucity or absence of circulating spherocytes. It is emphasized that because of some exceptions to this general pattern our conclusions are tentative and subject to confirmation. These exceptions may have been the result of several factors, e.g., the lack of a consistent time relationship between the blood findings (spherocytosis, osmotic fragility) and splenectomy; the influence of massive blood transfusions upon cord congestion; the effect of congestive cardiovascular failure upon the blood content of the splenic sinuses. The red cell content of the sinuses may also depend upon the sequence in which the splenic vessels are ligated in the course of splenectomy. Some surgeons ligate artery and vein simultaneously; others ligate the artery first, permitting the veins to empty to some extent. How much this contributes to the microscopic picture of relatively empty sinuses in spleens removed surgically is difficult to assess. However, a similar picture was seen in several spleens removed at necropsy and in such instances could hardly be an artifact.

In further studies we attempted to determine whether our observation might elucidate the rôle of the spleen in AHA and the variable response to splenectomy of patients with this disease. It occurred to us that if selective retention and destruction of spherocytes in the spleen were the main causes of the anemia in hereditary spherocytosis, the same mechanism might be at least a contributory factor in those instances of AHA in which spherocytosis is a prominent feature. Moreover, since not only the patient's cells are spherocytic, but in active cases the donated cells may also be rapidly converted into spherocytes, trapping of such cells in the spleen might seriously impair the effectiveness of blood transfusions.

It is conceivable that hemolysis in AHA occurs in two different ways: (1) The red blood cells may be damaged by auto-antibodies without necessarily becoming spherocytic and are either hemolyzed intravascularly⁴⁰ or phagocytized not only in the spleen but in other organs of the reticulohistiocytic system; (2) spherocytosis may be so severe that the anemia may be due primarily to selective trapping,

stagnation and lysis of the abnormal erythrocytes in the splenic cords. A combination of these two mechanisms may be responsible for the anemia in some, if not many, of the patients. Theoretically, then, splenectomy might have a more beneficial effect if decreased survival time of the red blood cells in a given patient with AHA results primarily from selective retention of spherocytes in the spleen rather than from intravascular hemolysis. With this idea in mind, we attempted to correlate the presence of spherocytosis and congestion of splenic cords with the hematologic and clinical response to splenectomy. However, careful analysis of hematologic and follow-up data failed to disclose any significant differences between the groups represented in Tables I to III. Neither spherocytosis nor congestion of splenic cords seemed to have any bearing upon the response to splenectomy or upon longevity.

The only other microscopic feature which we attempted to correlate with clinical and hematologic data was the presence of extramedullary hematopoiesis. It occurred in 40 per cent of patients in whom nucleated red cells were found in the blood. We demonstrated that patients with the more severe anemias were most likely to have erythroblastosis and extramedullary hematopoiesis. The median red blood cell count in this group was 1.0 million, with a range of 0.5 to 2.1 million. There was no apparent correlation between the severity of the anemia and the degree of erythroblastosis and extramedullary hematopoiesis within this group. Since some observers believe that the presence of nucleated red cells in the blood is a bad omen,⁴¹ we attempted to determine whether this were true for patients with AHA. The results are summarized in Table VI. Because the number of patients who had neither extramedullary hematopoiesis nor erythroblastosis was small and because death could not be attributed primarily to hemolytic anemia in three of the five fatal cases in this group (group III, Table VI), a valid comparison between patients with and without nucleated red cells in the blood could not be made. However, our findings would suggest that in AHA the presence of nucleated red cells in the blood does not have bad prognostic implications unless it is associated with extramedullary hematopoiesis. As pointed out previously, extramedullary hematopoiesis occurred in patients with the more severe anemias, which may account for the high mortality in this group.

Only one of 15 patients with extramedullary hematopoiesis had symptomatic hemolytic anemia secondary to malignant lymphoma. This case is not included in Table VI. It is conceivable that pa-

tients with malignant lymphomas, because of their impaired reticulo-endothelial system, usually do not respond to the stimulus of severe anemia with extramedullary hematopoiesis.

None of the other structural changes in the spleen lent themselves to clinicopathologic correlations from which useful information could be gained, but certain alterations in other organs are worthy of note.

TABLE VI
*Nucleated Red Cells in the Peripheral Blood in
Patients with Auto-immune Hemolytic Anemia
Not Associated with Malignant Lymphoma*

Groups under consideration	Number			Death primarily due to hemolytic anemia	Alive	Dead	Median red blood cell count
	Total	Alive	Dead		%	%	millions
I. Patients with nucleated red cells in the peripheral blood with extramedullary hematopoiesis	9	3	6	6	33	67	1.0
II. Patients with nucleated red cells in the peripheral blood without extramedullary hematopoiesis	14	11	3	3*	79	21	1.6
III. Patients with neither nucleated red cells in the peripheral blood nor extramedullary hematopoiesis	8	3	5	2	37	63	1.7

* Two of the three patients had hypocellular bone marrow.

Clinical hepatomegaly usually was explained on the basis of acute and chronic passive congestion due to secondary cardiac failure. Cardiac hypertrophy and dilatation were attributed to increased cardiac output as well as to myocardial injury occurring in patients with severe anemia. The degree of cardiac enlargement probably was related to both the duration and the severity of the anemia. In this connection it is significant that 13 of 27 patients in whom the immediate cause of death was established with some degree of certainty probably died from cardiac complications of the anemia. The average weight of the heart of 12 of these patients was 470 gm. (range, 345 to 600 gm.); in nine of these the heart weighed over 400 gm. In contrast, in 11 patients in whom the immediate cause of death was not attributable primarily to myocardial failure, the average weight of the heart was 364 gm. (range, 210 to 625 gm.) with only one weighing more than 400 gm.

Our studies indicate a definite relationship between the severity of the anemia nearest the time of death and the post-mortem findings of cardiac failure with pulmonary edema or central hepatic necrosis,

or both. Of 13 patients who died from cardiac failure in the absence of intrinsic cardiac disease, only two had a red blood cell count above 1.5 million and none above 2.0 million. In contrast, of ten patients who died from causes other than cardiac failure or acute hemorrhage, only one had a red blood cell count below 2.5 million and none below 1.5 million. Thus in patients with the more severe anemias the pathologic findings suggest the following mechanism of death: anemia \rightarrow myocardial hypoxia \rightarrow congestive heart failure \rightarrow pulmonary edema \rightarrow death. Of the vital organs the myocardium is probably the most sensitive to the hypoxia of severe chronic anemia. Central hepatic necrosis occurred in six patients with cardiac failure in the absence of intrinsic cardiac disease. In three of these the red blood cell count was below 1.0 million and in the remaining three it did not exceed 1.4 million. The possible impairment of erythropoietic function of the bone marrow by oxygen lack has been suggested on physiologic grounds,⁴² but no morphologic alterations indicative of this were demonstrable in most instances.

SUMMARY

Surgical and necropsy material derived from 50 patients with auto-immune hemolytic anemia (AHA) was reviewed and the pertinent gross and microscopic findings were described in detail.

The principal structural alterations were found in the spleen. Congestion of the splenic cords with relatively empty sinuses, a characteristic feature of hereditary spherocytosis, was found in only 36 per cent of the patients with AHA. In contrast to hereditary spherocytosis, hemosiderosis was severe in all cases and erythrophagocytosis frequently was present.

There was a high degree of positive correlation between spherocytosis and congestion of the splenic cords. This correlation was most consistent in patients with marked increase of the osmotic fragility of red blood cells. In contrast, congested sinuses with few red blood cells in the cords were invariably seen in patients without spherocytosis. These observations suggest that congestion of splenic cords in AHA is due largely to selective retention of morphologically abnormal red blood cells.

The morphologic observations in AHA with spherocytosis support the concept that selective retention of spherocytes in the splenic cords is a factor contributing to the patient's anemia. However, comparison of patients with and without spherocytosis by careful analysis of hematologic data and clinical follow-up studies failed to furnish conclusive evidence that the existence or degree of spherocytosis had

any bearing upon the patient's hematologic response or longevity following splenectomy.

Extramedullary hematopoiesis occurred in 15 patients (30 per cent). Only one of them had malignant lymphoma. Extramedullary hematopoiesis usually was observed in patients with the more severe anemias. Frequently it was associated with the presence of nucleated red cells in the blood. On the other hand, in many patients who had nucleated red cells in the blood, extramedullary hematopoiesis could not be demonstrated.

The bone marrow in AHA usually showed erythroid hyperplasia. In some instances, however, the bone marrow was hypocellular, indicating that sometimes AHA may be associated with anemia of a hypoplastic type.

The cardiovascular system may be greatly affected by the severity and persistence of the anemia. Cardiac enlargement was frequent and death from secondary cardiac failure occurred in almost 50 per cent of the cases in which the cause of death could be established. In these instances pulmonary edema and central hepatic necrosis were frequently observed.

We wish to express our appreciation to Ethel B. Hicks for her assistance in the compilation of data and statistical analysis of the material upon which this report is based, and to Helen Knight Steward for her editorial suggestions.

REFERENCES

1. Oettinger, W. Sur un cas d'ictère d'origine hémolytique non congénital; étude des lésions anatomiques. *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1908, 26, 391-408.
2. Micheli, F. Unmittelbare Effekte der Splenektomie bei einem Fall von erworbenem hämolytischen splenomegalischen Ikterus Typus Hayem-Widal (Spleno-hämolytischer Ikterus). *Wien. klin. Wchnschr.*, 1911, 24, 1269-1274.
3. Roth, O. Zur Frage des "Ictère hémolysinique" (Chauffard). *Deutsches Arch. f. klin. Med.*, 1913, 110, 77-89.
4. Eppinger, H. II. Erworbenener hämolytischer Ikterus (Typus Hayem). In: *Die Lehre vom menschlichen hämolytischen Ikterus, die hepatolienalen-Erkrankungen. Enzyklopaedie der Klinischen Medizin und der Speziellen Therapie*, J. Springer, Berlin, 1920, pp. 204-240.
5. Dick, W. Die histologischen Befunde bei einem Fall von hämolytischem Ikterus. *Med. Klin.*, 1925, 21, 1309-1311.
6. Davidson, L. S. P. Macrocytic haemolytic anaemia. *Quart. J. Med.*, 1932, 25, 543-578.
7. O'Donoghue, R. J. L., and Witts, L. J. The acute haemolytic anaemia of Lederer. *Guy's Hosp. Rep.*, 1932, 82, 440-456.
8. East, T. Acholuric jaundice with almost normal erythrocyte fragility. *Proc. Roy. Soc. Med.*, 1932-33, 26, 365-366.

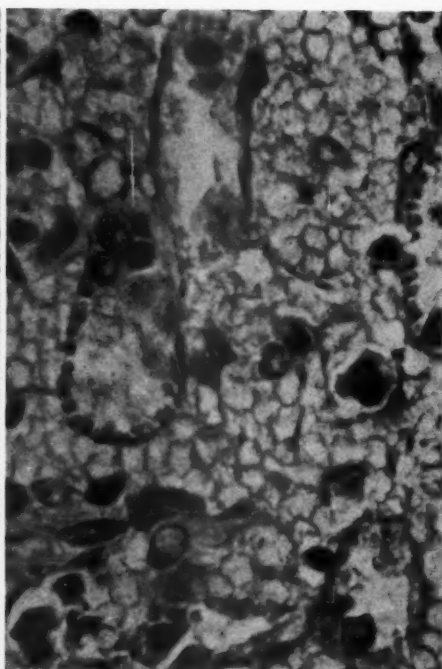
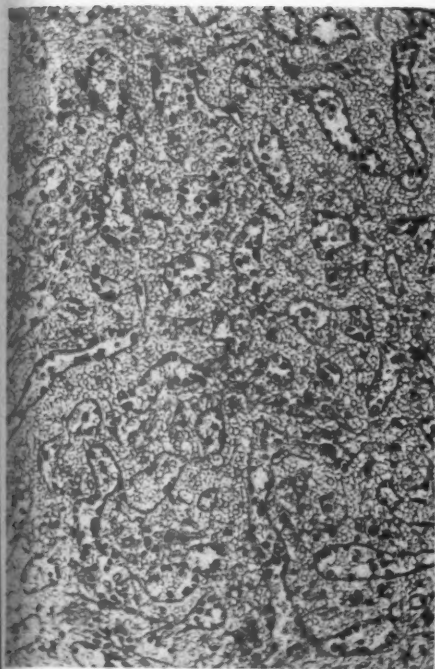
9. Kremer, M., and Mason, W. H. Acholuric jaundice in the adult. *Lancet*, 1936, 2, 849-852.
10. Rosenthal, F., and Corten, M. Über das Phänomen der Autohämagglutination und über die Eigenschaften der Kältehämagglutinine. *Folia haemat.*, 1937, 58, 64-90.
11. Israëls, M. C. G., and Wilkinson, J. F. Haemolytic (spherocytic) jaundice in the adult. *Quart. J. Med.*, 1938, 31, 137-150.
12. Rastetter, J. W., and Murphy, F. D. Acquired hemolytic jaundice. *Am. J. Digest. Dis. & Nutrition*, 1938, 4, 805-810.
13. West-Watson, W. N., and Young, C. J. Failed splenectomy in acholuric jaundice, and the relation of toxæmia to the haemolytic crises. *Brit. M. J.*, 1938, 1, 1305-1309.
14. Dameshek, W., and Schwartz, S. O. Acute hemolytic anemia (acquired hemolytic icterus, acute type). *Medicine*, 1940, 19, 231-327.
15. Farrar, G. E., Jr.; Burnett, W. E., and Steigman, A. J. Hemolysin anemia and hepatic degeneration cured by splenectomy. *Am. J. M. Sc.*, 1940, 200, 164-172.
16. Berrey, B. H., and Watson, J. D. Acquired hemolytic anemia: Report of case in an infant with discussion of several etiological factors which may have been operative. *Arch. Pediat.*, 1951, 68, 10-27.
17. Crosby, W. H., and Rappaport, H. Autoimmune hemolytic anemia. I. Analysis of hematologic observations with particular reference to their prognostic value. *Blood*, 1957, 12, 42-55.
18. Gridley, M. F. A modification of the silver impregnation method of staining reticular fibers. *Am. J. Clin. Path.*, 1951, 21, 897-899.
19. Snook, T. The guinea-pig spleen. Studies on the structure and connections of the venous sinuses. *Anat. Rec.*, 1944, 89, 413-427.
20. Gomori, G. Microscopic Histochemistry. The University of Chicago Press, Chicago, 1952, p. 36.
21. Motulsky, A. G.; Crosby, W. H., and Rappaport, H. Hereditary nonspherocytic hemolytic disease. A study of a singular familial hemolytic syndrome. *Blood*, 1954, 9, 749-772.
22. Meulengracht, E. Der chronische hereditäre hämolytische Ikterus (konstitutionelle Hypersplenie). Werner Klinkhardt, Leipzig, 1922, pp. 81-92.
23. Guizzetti, P. Hämolytischer, kongenitaler Ikterus. *Beitr. z. path. Anat. u. z. allg. Path.*, 1912, 52, 15-50.
24. Klemperer, P. The Spleen. In: Handbook of Hematology, Downey, H. (ed.). Paul B. Hoeber, Inc., New York, 1938, 3, 1722-1725.
25. Herzheimer, G. Ueber das Verhalten der kleinen Gefäße der Milz. *Berl. klin. Wchnschr.*, 1917, 54, 82-84.
26. Matsuno, G. Über Wandveränderungen der kleinen Milzarterien. *Virchows Arch. f. path. Anat.*, 1922-23, 240, 69-80.
27. Rappaport, H., and Johnson, F. B. Intracellular protein resembling Russell bodies in malignant lymphomas associated with acquired hemolytic anemia. *Blood*, 1955, 10, 132-144.
28. Whipple, A. O. Recent studies in the circulation of the portal bed and of the spleen in relation to splenomegaly. (Thomas Dent Mütter lecture.) *Tr. & Stud., Coll. Physicians, Philadelphia*, 1940-41, 8, 203-217.

29. Wintrobe, M. M. *Clinical Hematology*. Lea & Febiger, Philadelphia, 1951, ed. 3, p. 590.
30. Björkman, S. E. The splenic circulation; with special reference to the function of the spleen sinus wall. *Acta med. Scandinav.*, 1947, Supp. 191, 1-89.
31. Emerson, C. P., Jr.; Shen, S. C.; Ham, T. H., and Castle, W. B. The mechanism of blood destruction in congenital hemolytic jaundice. *J. Clin. Investigation*, 1947, 26, 1180.
32. Young, L. E.; Christian, R. M., and Izzo, M. J. Some newer concepts of "congenital" and "acquired" hemolytic anemias. *M. Clin. North America*, 1951, 35, 571-585.
33. Young, L. E.; Platzer, R. F.; Ervin, D. M., and Izzo, M. J. Hereditary spherocytosis. II. Observations on the role of the spleen. *Blood*, 1951, 6, 1099-1113.
34. Thompson, W. P. The splenic lesion in hemolytic jaundice. *Bull. Johns Hopkins Hosp.*, 1932, 51, 365-371.
35. Vaquez, H., and Giroux. Ictère chronique acholurique avec splénomégalie; ses relations avec l'anémie hémolytique. *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1907, 24, 1184-1192.
36. Gandy and Brulé. Ictère hémolytique congénital; autopsie. *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1909, 28, 369-375.
37. Paris and Giroux. Étude anatomo-pathologique d'un cas d'ictère hémolytique congénital. *Arch. d. mal. du coeur*, 1910, 3, 668-672.
38. McNee, J. W. The spleen: its structure, functions, and diseases. *Lancet*, 1931, 1, 1063-1070.
39. Turnbull, H. M. *Pathological Anatomy*. In: *The Anaemias*, Vaughan, J. M. Oxford University Press, London, 1934, pp. 188-200.
40. Loutit, J. F., and Mollison, P. L. Haemolytic icterus (acholuric jaundice), congenital and acquired. *J. Path. & Bact.*, 1946, 58, 711-728.
41. Schwartz, S. O., and Stansbury, F. Significance of nucleated red blood cells in peripheral blood. Analysis of 1,496 cases. *J. A. M. A.*, 1954, 154, 1339-1340.
42. Crosby, W. H., and Rappaport, H. Reticulocytopenia in autoimmune hemolytic anemia. *Blood*, 1956, 11, 929-936.

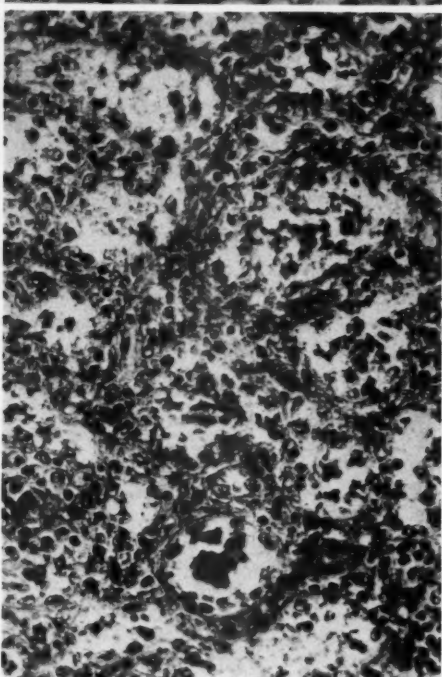
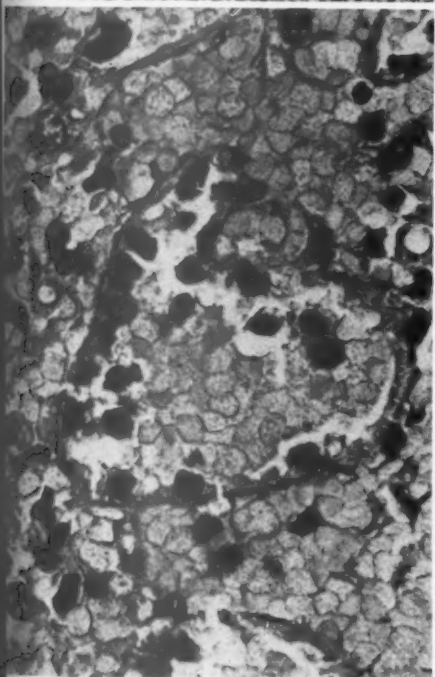
[Illustrations follow]

LEGENDS FOR FIGURES

- FIG. 1. Spleen (Armed Forces Institute of Pathology Accession 290203). Auto-immune hemolytic anemia (AHA) with spherocytosis. Of note are the marked congestion of the splenic cords and the comparatively few red blood cells in the sinuses. Hematoxylin and eosin stain. $\times 160$.
- FIG. 2. Same as Figure 1, higher magnification. The stomas between the lining cells (rod cells) are clearly visible in the cross section of a sinus. $\times 1200$.
- FIG. 3. Spleen (A.F.I.P. Acc. 486186). AHA without spherocytosis. Sinuses and splenic cords show congestion of about equal severity. Hematoxylin and eosin stain. $\times 900$.
- FIG. 4. Spleen (A.F.I.P. Acc. 594007). AHA without spherocytosis. The splenic cords are narrow and devoid of red blood cells; the sinuses are distended and some of them contain agglutinated red blood cells. Hematoxylin and eosin stain. $\times 300$.

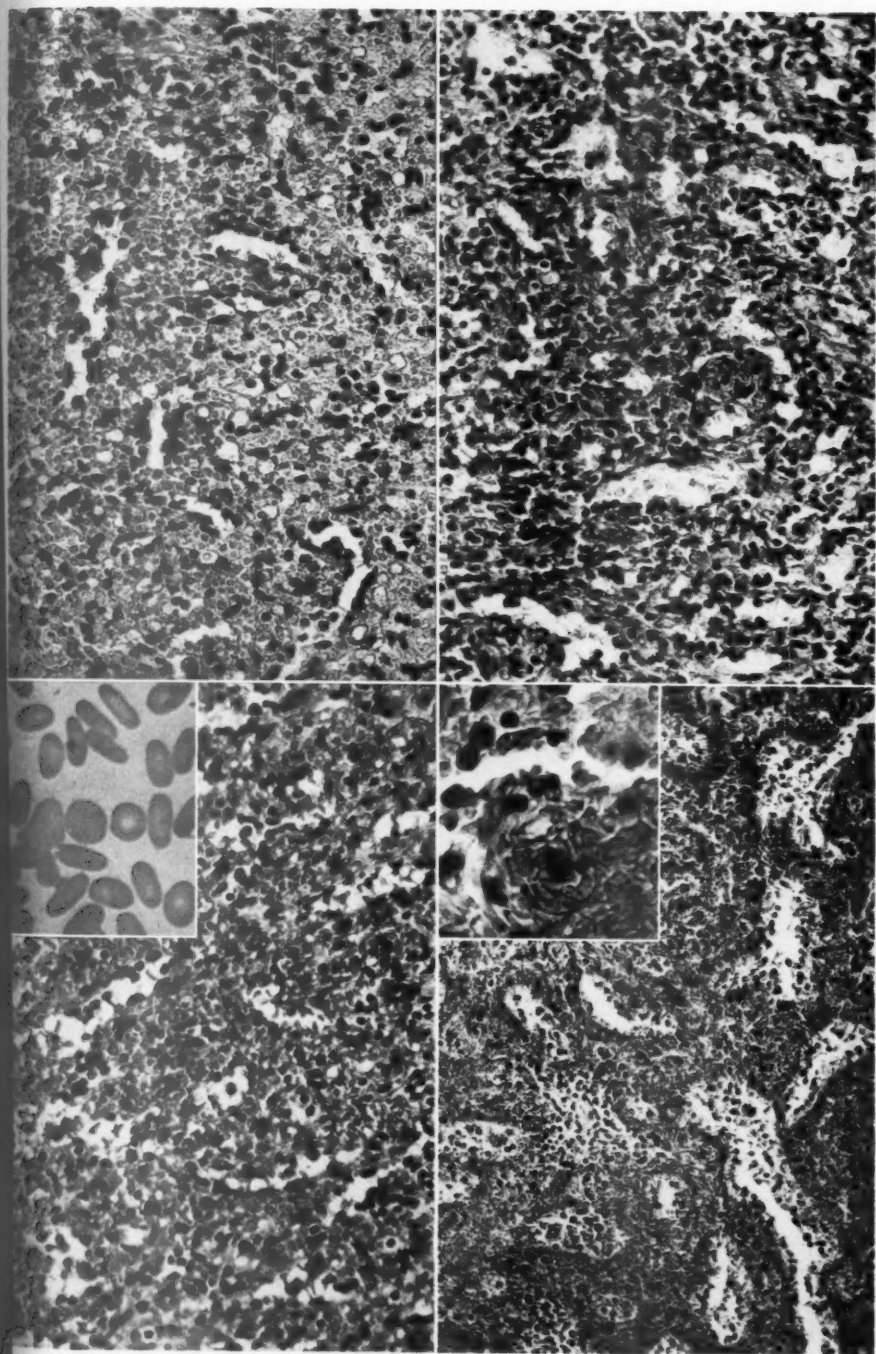


2



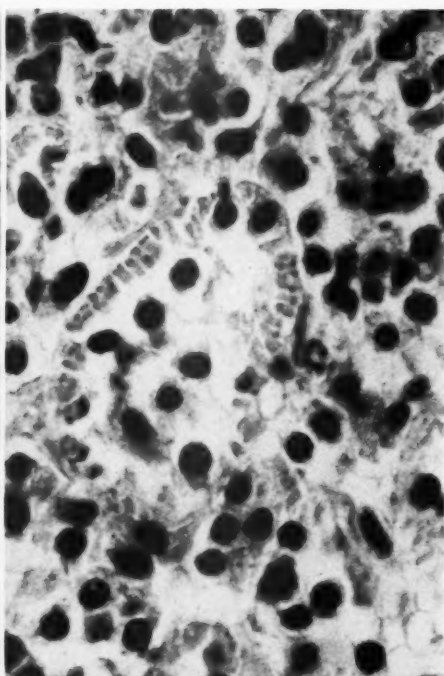
4

- FIG. 5. Spleen (A.F.I.P. Acc. 545885). Hereditary spherocytosis. Marked congestion of the splenic cords with almost empty sinuses and hyperplasia of the lining cells. Hematoxylin and eosin stain. $\times 300$.
- FIG. 6. Spleen (A.F.I.P. Acc. 496888). Hereditary non-spherocytic hemolytic disease. Absence of congestion of the splenic cords. Hematoxylin and eosin stain. $\times 300$.
- FIG. 7. Spleen (A.F.I.P. Acc. 639894). Hereditary ovalocytosis. Its similarity to the picture of hereditary spherocytosis may be noted. Hematoxylin and eosin stain. $\times 300$. (Insert: blood, Wright stain. $\times 900$.)
- FIG. 8. Spleen (A.F.I.P. Acc. 604224). Sickle cell disease. Six-month-old child who died after a period of severe anoxemia due to pulmonary tuberculosis. Marked congestion of the splenic cords. Hematoxylin and eosin stain. $\times 160$. (Insert, $\times 900$.)

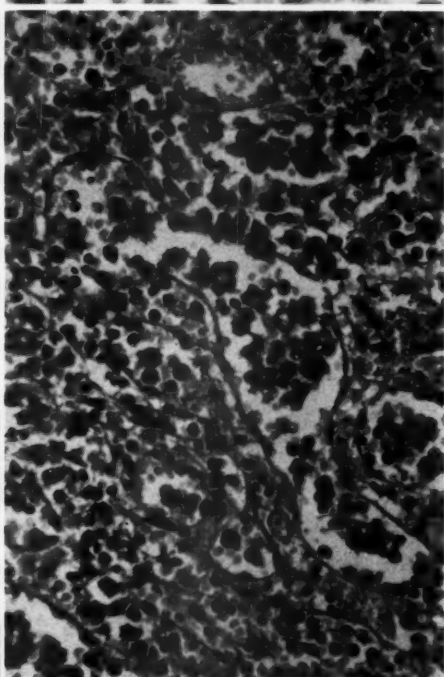
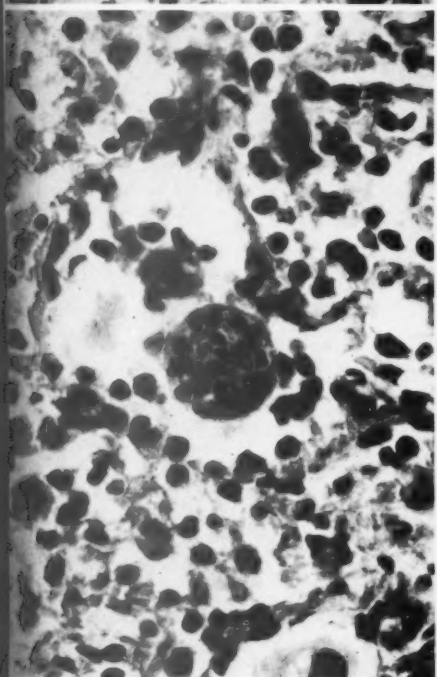


- FIG. 9. Spleen (A.F.I.P. Acc. 626829). AHA without spherocytosis. Tangential cut through distended sinus showing the stomas between the lining cells. Silver impregnation for reticulum (Snook) counterstained with Kernechtrot. $\times 800$.
- FIG. 10. Spleen (A.F.I.P. Acc. 626829). AHA without spherocytosis. Cross section of sinus showing the cytoplasmic bodies of the lining cells, most of them devoid of nuclei at the level sectioned. The stomas between the lining cells are clearly visible. The hourglass-shaped nucleus suggests passage of a macrophage through a stoma. Hematoxylin and eosin stain. $\times 1200$.
- FIG. 11. Spleen (A.F.I.P. Acc. 626829). AHA without spherocytosis. A macrophage packed with intact red blood cells lies within one of the sinuses. Hematoxylin and eosin stain. $\times 800$.
- FIG. 12. Spleen (A.F.I.P. Acc. 127761). AHA with severe extramedullary hematopoiesis, predominantly erythroid. Hematoxylin and eosin stain. $\times 300$.



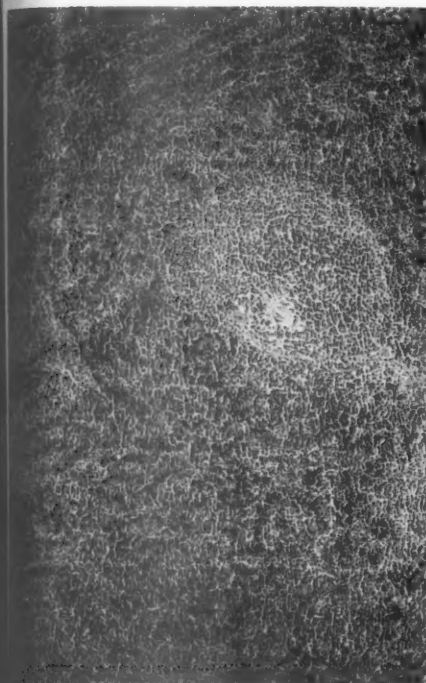


10

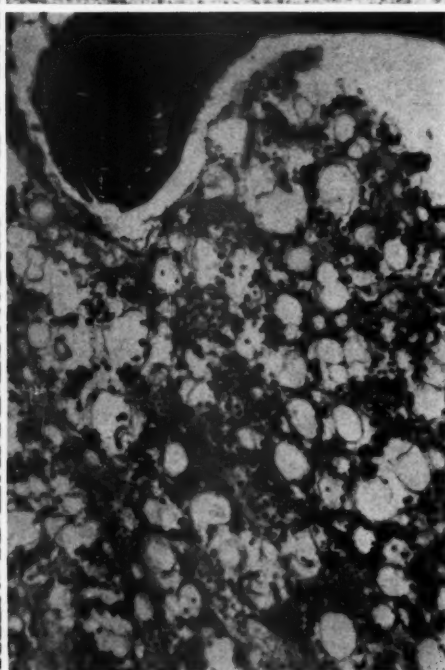
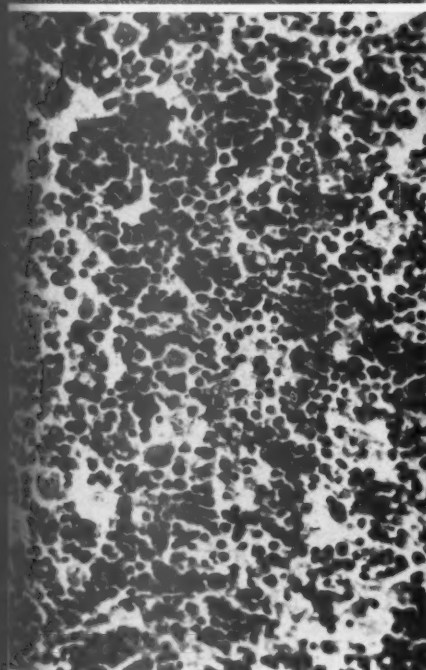


12

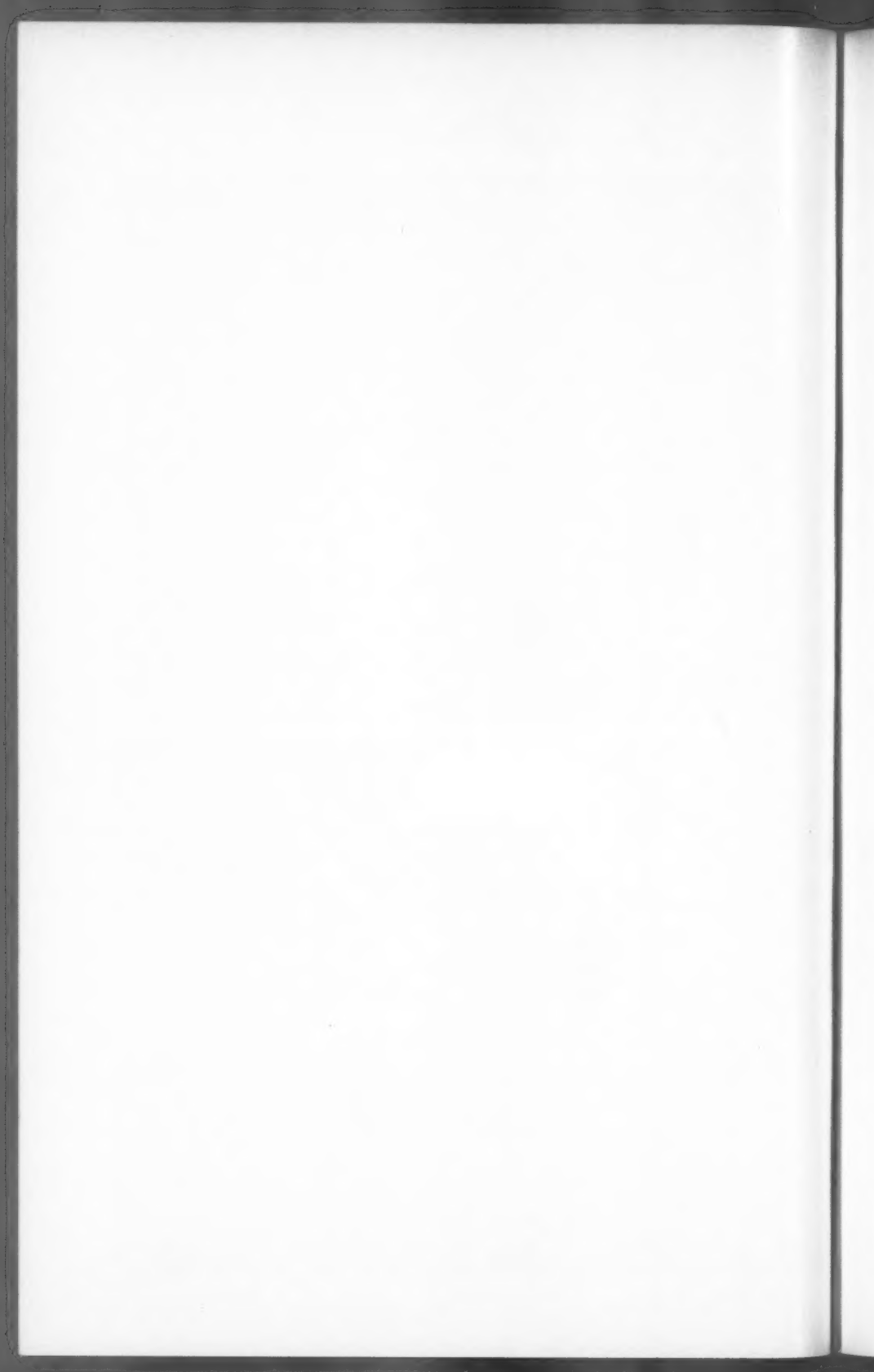
- FIG. 13. Spleen (A.F.I.P. Acc. 172123). AHA. Distribution of the iron-containing pigment with sparing of lymphatic follicles may be noted. Prussian blue method (Gomori). $\times 75$.
- FIG. 14. Spleen (A.F.I.P. Acc. 275088). AHA. The trabeculae and thickened reticulum fibers are impregnated with iron-positive material. Prussian blue method (Gomori). $\times 125$.
- FIG. 15. Bone marrow (A.F.I.P. Acc. 311839). AHA. Marked erythroid hyperplasia with relative paucity of granulocytes and megakaryocytes. The large pale cells are macrophages in which hemosiderin was demonstrated. Hematoxylin and eosin stain. $\times 300$.
- FIG. 16. Bone marrow (A.F.I.P. Acc. 523966). AHA. Cellular depletion of marrow, observed in three patients at necropsy. Hematoxylin and eosin stain. $\times 140$.



14



16



REGENERATION AND MALFORMATION IN THE NERVOUS SYSTEM,
EYE, AND MESENCHYME OF THE MAMMALIAN EMBRYO
AFTER RADIATION INJURY*

SAMUEL P. HICKS, M.D.; BARBARA L. BROWN, A.B., and CONSTANCE J. D'AMATO, B.S.

*From the Departments of Pathology, New England Deaconess Hospital
and Harvard Medical School, Boston, Mass.*

Much of our information about regeneration and abnormal development, especially of the nervous system, is based on experimental embryology of lower vertebrates and birds while less is known firsthand about mammals. Ionizing radiation has become a useful tool for studying the mechanisms of malformation in mammals because of its selective destruction of differentiating non-mitotic embryonal cells. By removing different members of the same litter at intervals starting immediately after the injury, the pathologic sequence can be followed accurately and the processes of abnormal development studied.

Abnormal development, obviously, is the result of deviation from normal ontogenetic pathways. The developmental pathways open to a given organism are primarily determined by the chromosomal makeup of the zygote but, like any system of chemical reactants, the genic material and its products are much influenced by surrounding factors. Deviation from the normal can then result from mutations (primary chromosomal alterations) or from interference with genetic processes already set in motion. For abnormalities produced by environmental shocks, such as temperature changes, that precisely imitated the effects of known mutations, Goldschmidt¹ coined the term phenocopy ("appearance copy"). Landauer² suggested that phenocopies may be not only the manifestation of altered normal pathways but the expression of mutations that would not appear unless pushed by the environment. Since all development is, in a sense, ultimately chromosome-controlled, the term phenocopy has come to be applied to virtually all malformations, on the assumption that for any environmental interruption of development there must be a combination of chromosomes that could do the same thing. Many things that happen experimentally and otherwise to embryos, ranging from minor extirpations and injuries to decapitation, result in abnor-

* This work was supported by Atomic Energy Commission Contracts AT (30-1) 1454 and 901, United States Public Health Service Grant B382, and the United Cerebral Palsy Association.

Received for publication, June 25, 1956.

malities that probably have no counterpart in mutations. For these and other reasons to be discussed, it seems prudent to categorize abnormalities into three groups as far as possible: those caused by (1) mutations, (2) phenocopies, and (3) by other forms of injury to the developmental processes.

The effects of radiation, in specified dose ranges, can be put into these same three categories, for radiation causes mutations and phenocopies⁸ and it can extirpate specific cells or parts of the embryo. It is the threefold purpose of this paper to demonstrate this extirpative effect, to illustrate by it that regeneration and malformation in mammals is basically similar to that in other vertebrates, and to add to the knowledge of the effects of radiation on embryos.

PREVIOUS EXPERIMENTS

Previous experiments^{4,5} have shown that malformations in rats induced by about 200 r. of conventional x-rays can be expressed in timetable fashion, relating the abnormalities to the stage of embryonic development. The severity of the pattern varied directly with the dose. The mechanisms responsible for this timetable have been partly worked out by looking at members of the same litter of embryos at successive intervals after the injury occurred rather than by simply guessing the age from the day of coitus. On the basis of past experiments and those about to be described, the timetable for 200 r. is something like the following. Severe head defects (anencephaly, pseudencephaly) result from radiation given in the presomite, early neural plate stage (late ninth day). Anophthalmia at about 4 somites and then microphthalmia a few somites later are initiated through the tenth day. There is a period between 10 and 20 somites when virtually complete recovery occurs. Encephalocele of the ventricles, and a recurrence of ocular defects, hydrocephalus, craniospinal abnormalities, and a number of visceral anomalies characterize the stage of about 20 somites (eleventh day), and certain cerebral deformities characterize the 30 somite stage (twelfth day). Syndactyly and certain defects of the forebrain are referable to the 40 somite stage (late 13th day) but, around 35 somites, animals relatively free of deformity may result. A whole series of abnormalities of the brain follows, collectively classed as microcephalies, decreasing in gross severity through about the 18th day with cerebellar anomalies beginning about the 13th day and increasing into the early neonatal period. At 200 r., skeletal deformities are relatively limited, but at 300 r., they are much more evident, as for example cleft palate and

nose, and a number of deformities of the limbs in the 30 to 40 somite stage (12th to 13th day).

MATERIALS AND METHODS

Albino rats of a colony originating from Wistar stock were used. They were kept in plastic cages, 70° to 85° F., with standard fluorescent lighting and little daylight. Purina Laboratory Chow pellets and water were fed ad libitum. Young adult female rats were placed with males and the day when spermatozoa were seen was counted the first day of gestation. In these experiments 200 r,* or occasionally 150 r., were given to the mother's whole body on a certain (estimated) day of gestation. Three to 4 hours after radiation some embryos were removed surgically, under ether anesthesia. Depending upon whether two, three, or four samples were planned, one half or a whole uterine horn was resected. This process was repeated at daily or greater intervals and spontaneous delivery was easy from one intact horn in experiments of two or three stages. Casualties involving the mother or young have been rare in a large series (some 200 operations) of such experiments.

All embryos and fetuses were serially sectioned after formalin or Bouin's fixation except in a few instances. Animals allowed to grow up were necropsied, pertinent organs studied microscopically, and the brain and cord were sectioned multiply or serially. Litters in these rats generally run remarkably uniform in age, almost to the somite. The principal material for this study (15 litters) is summarized in Table I. An additional 10 litters (not in Table I) were studied grossly and microscopically in two stages following radiation in the presomite to the 14 somite stage to learn more about the possible effects on inductive processes involving the forebrain and eyes. A large number of previous experiments^{4,5} was drawn on for comparison.

Members of a litter irradiated at 18 somites, for example, are called 18 somite animals. The group of litters irradiated at ages 18 to 24 somites may be more generally characterized as 20 somite animals or 11 day embryos. The term somite age is a convenient marker to epitomize stages of development.

Patterns of malformation often are summarized in names such as anencephaly, encephalocele, anophthalmia, syndactyly, hydrocephalus, and cleft palate but, like somite age, they will be qualified by further description.

* Two hundred and fifty kv., 15 ma., 70 cm., 3 mm. A1 inherent filter, General Electric Maximar therapy unit checked with a Victoreen r-meter and an integrating dosimeter.

The words regeneration (re + genere: to beget again, or renew lost parts) and restitution (re + statuere: to put back, repair, or restore) are used rather literally and interchangeably.

TABLE I
Summarization of Experimental Material (15 Litters)

Experiment no.	Somite age	Days of gestation										Post-natal	
		11	12	13	14	15	16	17	18	19	20		21
55-31	18	2	3	1	2								
54-7	19	6								1			
54-5	19	2						9					
54-6	22	3*							3				
56-6	24	4				3†					3		
56-5	24	3				3					3		
54-11	30		4						4				
55-29	30		4	2	2	3							
54-31	30		2					3				5	
55-30	32		2					2	2			3	
55-55	34			3									3
54-16	36			4				5					
54-15	36			3				5					
56-1	37			2			3						3†
55-32	41			2	3	2	3						

Column heads indicate the experiment number, age in somites at time of radiation, and the estimated days of gestation when members of a litter were sampled. The numbers in the days of gestation columns indicate how many young were removed. The last column indicates that the last sample was made some months after birth.

* One resorbing.

† All damaged during previous operation.

‡ One died at birth, no necropsy.

RESULTS

Our results involve considerably more than 100 serially sectioned animals and others studied less completely, so that an arbitrary selection of material to present in detail must be made. A brief reminder of what the normal embryos look like at 11 to 13 days will be followed by an account of the acute pathologic changes, the malformations that result, and the regenerative processes.

Normal Embryos

The normal rat embryo of 18 somites is just beginning to show cerebral vesicles and anterior limb buds. By 24 somites the cerebral vesicles are fairly prominent and the optic tubes are slightly concave

at the ends underlying the lens rudiment. The anterior limb buds are distinct. The heart has become a nearly complete spiral loop. The nephrogenic material, just a cord of condensed cells at 8 somites, is now a duct with a few tubules and will reach the cloaca at 32 somites. Gonads are first perceptible at 25 somites. At 30 somites the embryo has four limb buds, the anterior more advanced. The cerebral vesicles, retina, lens, and Rathke's pouch are distinct. The facial processes are developing well forward. Within, the neuraxis is assuming the configuration of multiple bends that characterizes it for the next several days and the abdominal viscera are beginning to be laid out, but the lungs are still only in bud form.

At 40 somites the embryo begins to look like an animal, the distal ends of the limb buds are flattening. The more anterior "somites" are becoming body segments microscopically. The face whose parts are not completely closed previews the adult. The brain is somewhat similar to that of 30 somites, but the parts are more clearly outlined. The striatum is a distinct mass ventrally in the forebrain and the tela choroidea is a recognizable inward cerebral fold. The bent mid-brain still forms the hump at the top of the animal and the flexed head is relatively enormous.

Acute Pathologic Effects

Two hundred r. of conventional x-rays quickly destroys a large proportion of the primitive non-mitotic differentiating cells throughout the embryo,^{4,5} while primitive mitotic cells or more differentiated ones are spared (Fig. 1). The threshold for this effect is about 30 to 40 r. when scattered susceptible cells are damaged. In neural-plate stages of the early somite it is more difficult to be certain what cells are killed by 200 r., for intermitotic cells and those just starting to differentiate are hard to distinguish. Three hours after radiation, however, the mitotic cells continue to be evident when they normally would be while neighboring cells are dead. Mesodermic cells under the plate show considerable destruction. At around 400 r. the intermitotic cells close to the neurectoderm as well as the mitotic layer begin to show damage, and 800 r. kills most of the least differentiated cells while further differentiated ones stand up and continue to develop for some days at least.

Eleven Day Embryos. In the 20 somite embryo, the neural tube has an actively mitotic inner cell layer and then outside of this is a layer of non-mitotic primitive differentiating cells, the future neurons and glia. These differentiating cells are the radiosensitive ones. At this stage normally the mitotic layer is approximately one or two cells

in thickness, the primitive differentiating layer is variable, being about four or five cells thick in the cerebral vesicles, and a little thicker laterally in the spinal cord. These differentiating cells, products of the mitotic neurectoderm, are virtually never in mitosis (even after experimental colchicine). Further maturation beyond this level is present only to a slight degree in the 20 somite embryo. The flat-celled layer of the roof of the hind brain and some of the very outermost layer of cells throughout the neuraxis represent a step beyond the primitive non-mitotic differentiating stage. The thin plates of epithelial cells at the dorsal and ventral aspects of the center of the spinal cord are another example of further differentiation beyond the primitive stage, for they are now non-mitotic, radioresistant, and really young ependymal glial cells.

Radiation injury was well developed in 4 hours and the dead cells constituted roughly from one third to two thirds of the neural cells in various regions. The affected cells were completely disintegrated by that time and were represented by clumped remnants of basophilic nuclear material or nuclei greatly reduced in size, deeply basophilic, and frayed at the margins. The normally scant cytoplasm of the cells was usually unrecognizable. Necrotic cells were desquamated and clumped in the cavities of the central nervous system, indicating that the neurectodermal layer was not a withholding membrane and that some cells in the radiosensitive stage were close to the surface. (In older embryos the inner surface remained more intact with less spilling of dead cells into the ventricular system.)

The cerebral vesicles were severely damaged, especially their anterior lateral and dorsal parts, but the anterior diencephalon was considerably less so except at the base of the optic tubes. Just caudal to this in the mid-brain, medulla, and cord, destruction again became notable. These vulnerable zones correspond to qualitatively vigorous mitotic activity in the adjacent neurectoderm. The tips of the optic tubes, actively becoming retinas, were extensively necrotic while the more proximal parts with fewer differentiating cells were less damaged.

Mesenchyme throughout the embryo was affected as were some other cells and it was apparently the non-mitotic primitive differentiating cells that were the ones destroyed. Pronounced damage occurred in the densely packed mesenchymal cells of the distal parts of the mandibular processes, those posterior to the tongue and heart, around the gut in the thoracic region. Some scattered necrotic cells were seen throughout the looser mesenchyme of the embryo and necrosis was marked in the somites. The dorsolateral cells that constitute the migrating sclerotome, as well as some cells of the outermost

cortex or cutis plate, were hit heavily. The zone of mitotic activity of the cortex and just inside it were largely spared. Cells of the inner core were damaged; but in older embryos when they elongated into primitive muscle they escaped injury. The dense mesenchyme at the root of the forming liver, around the lower gut, along the nephrogenic cord, and more laterally in the body wall adjacent to the stalk was heavily damaged. The mesenchyme of the tail bud just under the thick ventral ectodermal ridge of Grüneberg was wholly necrotic but the ectoderm was intact. Occasional epithelial cells of the nephrogenic cord might have been affected, but so severe was the mesenchymal necrosis around them that this was uncertain. In the heart, vessels, ectoderm, extra-embryonic membranes, and placental tissue, no visible damage occurred. The otic (ear) vesicle showed some destruction. The mesenchyme around the retinal tip of the optic tubes and some cells of the thickening lens plate were dead. Mesenchymal cells forming the base of the limb bud were heavily damaged also, but the epithelium, including the ridge over it, was unscathed.

Twelve Day Embryos. Damage at 30 somites was analogous to that at 20. More cells had passed the primitive differentiating radio-sensitive stage in many tissues, yet abundant early differentiation and proliferation were going on in other regions such as the skeletal anlagen and nervous system. The cerebral vesicles, except their medial aspects, were especially damaged but the diencephalic walls just behind the vesicles were much less affected. Around the base of the optic tubes and in the mid-brain, destruction was considerable. The posterior mid-brain, posterior half of the medulla, especially laterally behind the wider part of the rhomboid region, and the differentiating layers of the cord were hard hit. Some damage to the lens was present, the differentiating concave layer of the retina facing it was severely affected, and so was the mesenchyme in the folds beginning to develop around the eyes. Some necrosis of cells of the otic (ear) vesicle occurred. Postcardiac mesenchymal cells, the mesenchyme at the tips of the first two lung buds, and the cores of the limb buds anterior to the vessels and nerves appearing in them were virtually decimated. The cores of the more anterior somites, now past the more primitive differentiating stages, were unaffected but their surrounding mesenchyme and the more primitive caudal somites were damaged as in earlier embryos. The bands of denser mesenchyme between the blocks of looser tissue that become vertebrae showed damage, but generally the notochord was spared. The nephric mesenchyme and some of the associated medially placed epithelial clusters showed some patchy destruction. The dense mesenchyme of the mesentery, lateral body

wall, roots of the limb buds, and of the nasal and facial processes was heavily damaged.

Thirteen Day Embryos. Four hours after radiation the destruction of cells in the 34 to 41 somite embryos was basically the same as in earlier ones. Damage was very severe in the dorsal parts of the cerebral vesicles, the differentiating layer of the retina, the dorsolateral part of the caudal medulla, and the mesenchyme in the lateral tips of the nares, maxillary folds and lateral jaw margins, and the peri-orbital mesenchyme. The striatal masses of the brain showed some damage to their population of primitive differentiating neural cells but the neurectodermal zone was not disrupted. The dense mesenchyme in the bases and distal ends of the limb buds, the tip of the genital tubercle, and the differentiating cells in the dorsal part of the spinal cord were severely damaged. Other mesenchymatous regions corresponding to those in earlier embryos showed some destruction. The mesenchyme of the folds that were to become a palate was moderately affected as was that of mesenteries and the perineal region. The nephric mesenchyme was virtually undamaged. Liver, heart, and lung bud structures were spared, but adjacent mesenchyme showed destruction of cells and some necrosis of cells occurred in the endocardial valve cushions in one animal. The vertebral structures were affected as in the 30 somite embryos. There was no vascular damage.

Malformations

Eleventh Day. The principal malformative patterns at the eleventh day were a dorsal encephalocele (or ventriculocele) due to outpocketing of the tela choroidea of the junction of the third and lateral ventricles (Fig. 3) and a short deformed tail. A deficient skull vault and edema usually accompanied the ventriculocele, and internal hydrocephalus sometimes occurred. Urogenital anomalies were frequent. The eyes were defective, ranging from distorted microphthalmia to absence. The brain stem and spinal cord showed abnormalities.

In more detail, the encephalocele was a backward dorsal protrusion of the junction of the roof of the third ventricle where it joins the lateral ventricles, associated with an oversized choroid plexus. In litters consisting of 19 and 22 somite embryos there was no skull vault and the meninges were continuous with the subcutaneous connective tissue. The top of the head was covered with a single layer of flat epithelial cells. The meningeal connective tissue was loose and distended by fluid which often spread through the subcutaneous tissues. The cells of the choroid plexus were properly oriented, but

the arachnoid space around the brain stem was large and filled with fluid. In the 24 somite embryos general edema was absent and the deficiency of the vault of the skull was much less apparent, there being no distinctive dome as in the embryos irradiated somewhat earlier. Nevertheless, the ventriculocoele was well developed.

The cerebral mantle sometimes was thinner than normal but with no jumbling of cells. When the animal was taken nearly at term, there was sometimes a dilatation of the lateral and third ventricles. Several examples of this occurred in the litters from 19 to 24 somites. The mid-brain, medulla, and spinal cord showed deformities characterized by rosettes of neurons and glia, or immature cells (Fig. 2) in the subependymal and ependymal regions, depending on the age of the animal examined. The medulla usually was reduced in bulk, with not so much a disturbance of cyto-architecture as a decrease in numbers and density of neurons in any given area. Occasional minute ectopic buttons of ependymal and subependymal cells protruded into the neural cavity from the floor of the medulla or from the wall of the aqueduct. In the 18 somite embryos (litter 55-31) the only abnormality was the formation of minute dorsomedial encephalocoeles in one of the two fourth stage embryos taken at 14 days.

The litters of 19 and 22 somite embryos showed consistently deficient or sometimes absence of lenses, retinas, and optic tubes or nerves (depending on the age when examined). There was sometimes no eyeball, but asymmetries of the defects were common and there was always some sort of an orbit. Nearly normal eyes occurred in some 24 somite animals, on one side, but so did extreme microphthalmia. In the 18 somite litter, the eyes had developed well throughout the period of serial sampling.

Urogenital changes were of interest because of the initial injury to the nephric mesenchyme. One of the nine members of the second stage of the 19 somite litter (54-5) had no left kidney, but bilateral gonads were present and the right kidney was set low with its artery and vein arising above the bifurcations of aorta and vena cava. Another had no left kidney, but its normal right kidney had two ureters, the lower one crossing over to the left side just above the bifurcation of the vena cava to descend to the urinary bladder. Both adrenal glands were present, but the left gonad was deficient, appearing as poorly defined tubules in a mesenchymal mass. The single fetus in litter 54-7 developed dilated ureters which had failed to communicate with the urinary bladder. Blind ureteral endings might have caused dilatation, but in some animals perfectly patent ureters were dilated.

Anomalies of the urinary tract occurred in both 24 somite litters (56-5 and 56-6). Three fetuses (56-5) had, respectively, unremarkable tracts, dilatation of both ureters, and a dilated left ureter. In litter 56-6 one fetus had a dilated left ureter while another had only a rudimentary left kidney.

Other visceral changes were rare. The aorta and pulmonary arteries had not quite separated just above the heart in one case. The left lung was placed more posteriorly than usual in some animals, scarcely more than is normally the case in rats. Umbilical herniation of abdominal viscera (including liver) was rare, being present in some members of litter 54-5. (The gut does not normally recede into the belly until the 18th day.)

Skeletal changes aside from the defective skull vault and the tail were rare or difficult to ascertain. Absent (cleft) palate, nasal-labial abnormalities, and deficiencies of the bony spinal arches were not seen in this study but appear at 11 days after higher doses. Despite the extreme deformity of the tail characteristic of this period, the perineal structures were not abnormal externally. No 11 day animals have survived after birth.

Twelfth Day. Malformations seen in the first few days of 12 day animals were distortion of the dorsal outer layers of the cerebral vesicles and a continuing tendency for the tela choroidea at the junction of the lateral and third ventricles to become involved along with the meninges of this region. Irregularities of the mid-brain and cord were conspicuous soon after radiation, but the diencephalon showed less, and skeletal and visceral anomalies virtually were absent.

Three 30 somite litters were sampled on the 17th or 18th days of gestation. In litters 54-11 and 54-31, seven fetuses showed a dense approximation of the meningeal fibrous tissue to the upper mid-brain and to the tela choroidea at the junction of the lateral and third ventricles. This was somewhat like the 11 day pattern in that the choroid of this region was prominent and there was some degree of outpocketing of the ventricular-choroid complex. Rosette formation of the cells of the cortex was somewhat variable in distribution and extent. These deformations were, as in those fetuses studied at earlier stages, variously predominant posterior, medial, or lateral to the dorsal central region of the cerebral hemisphere, and in two fetuses distortion by rosettes was surprisingly minimal. The eyes were normal. At 21 days five fetuses were examined from litter 54-31. The deeper portions of the dorsal cerebral cortex, the inner half approximately, were involved by rosette deformities, and this extended medially to

the hippocampus. There was a thickened connective tissue vault over the third ventricle and in one animal this was accompanied by a slight upward protrusion of the tela choroidea. Medullas somewhat smaller than normal, corresponding to those described above, with rosette distortion of the posterior subependymal portions, were present. The eyes were equivocally smaller than normal in some but normal in size in others. In two animals the retina of one eye showed a single lateral rosette. Other anomalies were absent except a dilated ureter close to the kidney in one case.

Thirteenth Day. The principal malformations that are known to result from irradiation at about 40 somites are deformed brains and feet. Irradiation at 34 and 37 somites (litters 55-55 and 56-1) did not result in abnormal feet or architectural deformations of the brain, but there was a variable reduction in total size of the brain independent of the animal's body size in two instances. This was striking when multiple sections of these brains from front to back were superimposed on corresponding normal sections. There seemed to be simply a little less of everything rather than focal deficiencies.

Two litters in the 13 day group were sampled on the 17th day of gestation and ten fetuses were obtained (litters 54-15 and 54-16). They had been irradiated at 36 somites. Both showed indistinct development of the front toes in some members by 17 days, although this was early to anticipate how poorly the feet might later develop. As in the 41 somite litter, rosettes were present in the dorsal, lateral, and inferior parts of the anterior cerebral vesicles and in the striatum. The roof region of the cerebellum and optic nerve head had some rosettes. Rosette deformities or slight irregularities persisted in the dorsal spinal cord plate extending to the meningeal surface, and in the dorsolateral medulla. The medulla, pons, and possibly some parts of the spinal cord were slightly reduced in bulk. Minute ventricular ectopias occurred in the medulla and mid-brain. A single animal had, in addition to the cerebral rosettes, a very mild version of the out-pocketing of the tela choroidea and its feet were forming irregularly. The final stage of the litters was taken when the palatal folds were still coming up from beside the tongue so that cleft palate could not be anticipated with certainty. Litters 55-55 and 56-1 (34 and 37 somites) showed closed palates.

In litter 55-32 (41 somites) radiation caused severe necrosis in the tips of the limb buds. The 24, 48, and 72 hour samples of the litter showed that this necrosis had persisted in almost all limbs as a cystic defect in the center of the paw (Fig. 4).

Other Experiments

The ten litters subjected to radiation from 0 to 14 somites and sampled twice indicated, as had been determined previously,^{4,5} that severe defects of the head, face, and brain resulted from damage to the earliest neural plate of the presomite stage. In these the prechordal mesoderm beneath the anterior plate was severely damaged as were some cells in the plate itself. Analogous damage with more neural destruction occurred at from 2 to 6 somites, yet defects were virtually limited to the eyes. The pituitary body sometimes dipped slightly into the sphenoid sinus. Radiation (150 to 200 r.) at 6 to 14 somites produced little or no malformation, the abnormality, when measurable, being limited to somewhat small eyes.

A review of early work indicated generally that nasolabial, palatal, and severe limb defects were more readily produced by 300 to 400 r. than by 200 r. in the 12 to 13 day period.

Regeneration

As Table I shows, litters were examined from 20 hours to several weeks after the first sample was made. Details of every litter cannot be given, so general aspects will be presented.

In 11 day litters the total damage sometimes ranged as high as one third to one half of the cells in the embryo and in later stages the number of damaged cells was less only because proportionately more cells had passed the radiosensitive stage. Yet recovery was the rule and only in certain circumstances did abnormality result. How did restitution come about? In the nervous system it was from the surviving mitotic neurectoderm, sometimes relatively simply and complete, sometimes complex and with varying degrees of abnormality. In the mesenchyme, unless the damage was drastic or involved a critical inductive process, the effects were quickly covered up by proliferation of the remaining mesenchyme. Sometimes even after decimation of a region, recovery was perfect. In the limb buds at 41 somites the mesenchyme was so sensitive to damage that a persistent defect resulted. The ectodermal ridge, that has to do with limb differentiation, was not visibly damaged by 200 r. and it seemed that the malformation was simply the result of a gross defect (Fig. 4). In one 13 day animal (litter 55-32, 48 hour sample) cystic defects in the muzzle appeared similar to those in the paws, but the faces of the littermates taken at 72 hours (16 days old) were not measurably abnormal.



The formation of rosettes is interesting and important and it deserves detailed consideration. When damage was severe, rosettes

formed secondarily as a result of destruction of the continuity of the neurectodermal layer. Even in 4 hours the orientation of these cells was mechanically disturbed and in the dorsal cerebral vesicles, brain stem, and cord, the mitotic layer of cells had begun to form clusters or rosettes. Formation of rosettes (a little rose or cluster of cells around a center) is a characteristic reaction in the embryonic nervous system, occurring after many injuries to the primitive neural cells (Fig. 2). If there is a simple explanation for the rosettes in these irradiated embryos, it lies in the fact that in normal growth the neurectodermal layer is maintained in continuity over a broad surface by several balanced factors. The inner ventricular pressure and the outer tissue pressure of the growing bulk of the primitive differentiating layer are in a continual though changing equilibrium. An orderly plan⁶ in the neurectoderm normally determines how many cells will move out into the differentiating layer after the mitotic divisions and how many will stay behind to expand the surface of the growing ventricular surface. When radiation kills the sensitive cells, the neurectodermal surface is secondarily affected by the collapse of this zone. An imbalance between the tissue pressure and ventricular pressure may be what makes the surface irregular and leads to clusters or rosettes of neurectoderm. Mitotic cells line the rosettes if they have a hollow center, or a few mitotic cells form the center if there is no obvious cavity. The cavities, at least in the earliest stage of malformation, are continuous with the main neural cavity, but subsequent growth may isolate them. They do not begin appreciably far from the ventricular surfaces and it does not appear that the differentiating layer of cells creates the new mitotic centers. From each of these disoriented centers primitive differentiating cells continue to be proliferated so that in time it is possible for a large multilayered rosette to be formed. Depending on the degree and extent and, probably especially, on the site and time of this initial disorientation, the subsequent disposition of neurons and glia derived from these cells can be either slightly disturbed, considerably mixed up, or almost all evidence of it can disappear completely. Rosettes in some degree are very common and profuse in most germinal zones shortly after radiation yet subsequently the architecture of the region for which they are responsible can often straighten itself out quite well.

Some specific aspects of the regenerative process are illustrated in Figures 2, 5, and 6 where early rosettes, their fate in the brain irradiated on the 13th day, and regeneration in the spinal cord are shown. In Figure 5 it is interesting to note that the original zone of proliferating cells has become a jumble of rosettes and is actually

a hypertrophied mass of "extra" brain tissue. Despite this chaos, cells migrated out from this source to produce a fairly orderly if not perfect outer cerebral cortex.

DISCUSSION

Three aspects of these (and previous) studies may be considered. One is the mechanism of radiation malformation, another is how good is radiation as an extirpative tool for experimental mammalian embryology, and the third is the problem of phenocopies.

Although 200 r. produce a highly selective necrosis of specific embryonal cells, the number and distribution of dead cells is usually far out of proportion to any malformation that might follow. The discrepancy is resolved by considering that the mammalian embryo is basically the same in its developmental processes and capacity to regenerate after injury as other vertebrates. Malformation is related to the complex balance between interference with key inductive and other growth processes and repair. It is concluded that severe cephalic defects (anencephaly) are chiefly referable to damage to the prechordal mesoderm under the neural plate in the presomite stage, and anophthalmia to interference with relations between the optic cup and ectoderm around 2 to 6 somites. Cell destruction of the selective type described is severe in the neural and mesenchymal tissues, but by itself does not explain the abnormal development.

On the eleventh day, damage is again disproportionate to what finally happens. Two points can be noted. The prospective cerebral roof—tela choroidea—is vulnerable to injury as early as 20 somites. The anophthalmia at this stage results from destruction of early lens and retina, structures already formed, in contrast to interruption of the induction stage of the eye earlier at 2 to 6 somites. Between 6 and 20 somites nearly normal to normal animals result after irradiation with 200 r.

There were three instances in which epithelial-mesenchymal relations were of interest although not wholly explained. In the 11 day animals severe destruction of nephric mesenchyme without evident damage to epithelium was associated with urinogenital anomalies. Apical ectoderm of limb buds escaped destruction at all stages, but malformation of feet occurred around 40 somites, apparently only in the presence of extraordinary destruction of mesenchyme that could not be compensated. The ectodermal ridge of the ventral tail bud, discovered recently by Grüneberg⁷ to be deficient in mice with mutant abnormalities of the tail, was spared by radiation in the 11 day period in which taillessness was a malformation. The immediately

underlying focal region of dense mesenchyme, however, was almost completely destroyed.

As a tool for experimental mammalian embryology, radiation shows promise as a means to investigate development of the nervous system and possibly the skeleton. If the ectoderm (epidermis) continues as resistant to higher doses as it is to 200 r., then the selective radionecrosis of mesenchyme may provide a way to explore skeletal development. The present study and a previous one⁵ have revealed that the early brain and spinal cord of rats have a capacity to regulate and regenerate comparable to that described in other vertebrates.⁸ Preliminary attempts have been made to correlate the vulnerability of various parts of the brain between 20 and 40 somites (and older embryos) to the proliferation and migration areas of Bergquist and Källén.⁶ These authors, in a series of studies, mapped out and sought to homologize the proliferation centers, and followed the successive migrations of neural cells from them, in a number of vertebrates. Vulnerability to damage by primary radiation and the malformation that results seems, on this preliminary basis, to be compounded of several factors. Some of them seem to be as follows. In a given region, destructive damage will be proportional to the number of cells in the radiosensitive stage. Restitution must come from the adjacent neurectodermal proliferating area and recovery may be determined by whether that area is still at peak mitotic activity or subsiding or resting prior to producing another crop of migrating cells. Migrating cells, too, may be in the radiosensitive stage or more mature. A complicating factor is the production of rosettes. As noted, this appears to be a deformity of the neurectoderm secondary to destruction of its proliferated cells that act as its mechanical tissue support. Even deformed neurectoderm seems capable, however, of going through its paces of producing waves of migrating cells. Figure 5 shows that although the neurectoderm was so disrupted at 13 days that its local architecture remained a chaos of rosettes, it still managed to get cells on the migratory paths to produce a recognizable cortex. A systematic study of normal mitotic and migratory patterns in the brains of 12 to 21 day old embryos and the effects of radiation on them is now in progress, and it should clarify some of these aspects concerning development of the central nervous system.

Finally, in respect to genetic aspects one does not expect mutations (with certain obvious exceptions) in a normal population to appear in all members of litters; and corresponding phenocopies, if they derive from mutations that need an environmental push for expression,² should not appear either. The radiation malformations that regularly

affect all members of a litter may be looked upon as reactions to interruption of developmental pathways normal to the species or, for that matter in many instances, normal to vertebrates. Where there are differences in response (some skeletal changes, for example³), not attributable to slight differences in age of the embryos, then genetic differences in susceptibility to injury or in developmental pathways must be called to account. Whether these differences are classified as due to mutations or variations from the chromosomal configuration which is normal for the species, is a matter of viewpoint. Experiments with varying doses and different strains and species may prove helpful.

REFERENCES

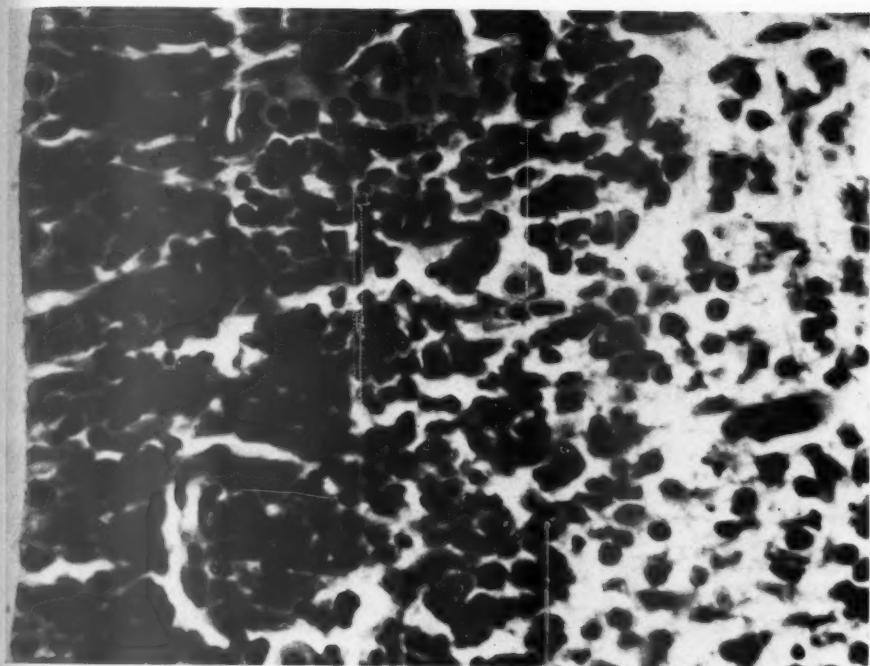
1. Goldschmidt, R. B. Theoretical Genetics. University of California Press, Berkeley, 1955, 563 pp.
2. Landauer, W. On the chemical production of developmental abnormalities and of phenocopies in chicken embryos. *J. Cell. & Comp. Physiol.*, 1954, **43**, Suppl. 1, 261-305.
3. Russell, L. B., and Russell, W. L. Changes in the relative proportions of different axial skeletal types within inbred strains of mice brought about by x-irradiation at critical stages in embryonic development. *Genetics*, 1950, **35**, 689.
4. Hicks, S. P. The effects of ionizing radiation, certain hormones, and radiomimetic drugs on the developing nervous system. *J. Cell. & Comp. Physiol.*, 1954, **43**, Suppl. 1, 151-178.
5. Hicks, S. P. Mechanism of radiation anencephaly, anophthalmia, and pituitary anomalies. Repair in the mammalian embryo. *A. M. A. Arch. Path.*, 1954, **57**, 363-378.
6. Bergquist, H., and Källén, B. Notes on the early histogenesis and morphogenesis of the central nervous system in vertebrates. *J. Comp. Neurol.*, 1954, **100**, 627-659.
7. Grüneberg, H. The developmental mechanisms of genes affecting the axial skeleton of the mouse. To be published.
8. Weiss, P. A. Nervous System (Neurogenesis). In: Analysis of Development, B. H. Willier; P. A. Weiss, and V. Hamburger (eds.). W. B. Saunders Co., Philadelphia, 1955, pp. 346-401.

LEGENDS FOR FIGURES

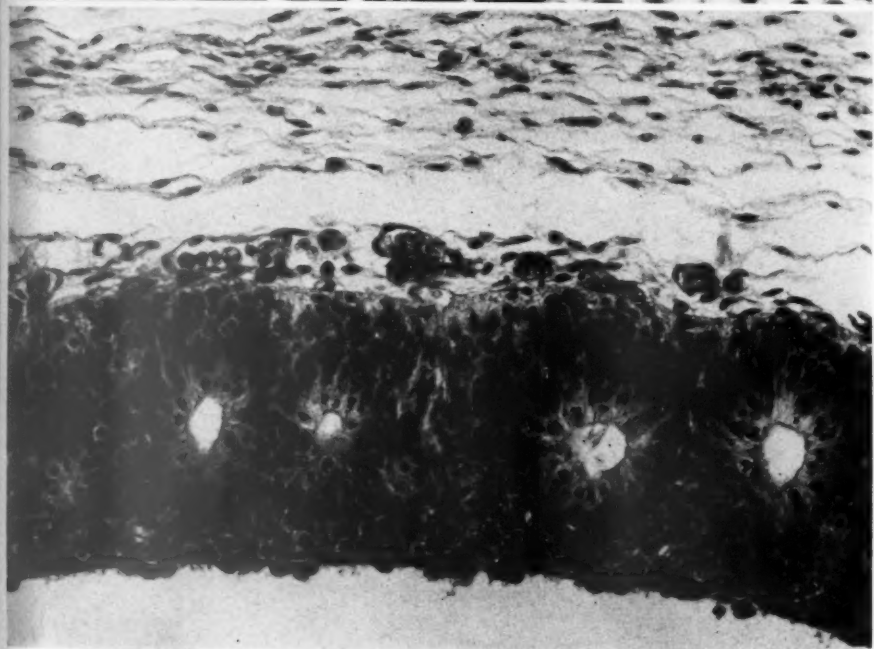
FIG. 1. Acute necrosis of primitive differentiating neural cells in the brain stem of a 12 day embryo 4 hours after administration of 200 r. Mitotic figures persist in the neurectoderm. Dead cells appear as compact black dots. Hematoxylin and eosin stain. $\times 700$.

FIG. 2. Rosettes in the wall of the cerebral vesicle of a fetus 48 hours after 150 r. were given on the 12th day. Hematoxylin and eosin stain. $\times 350$.





1



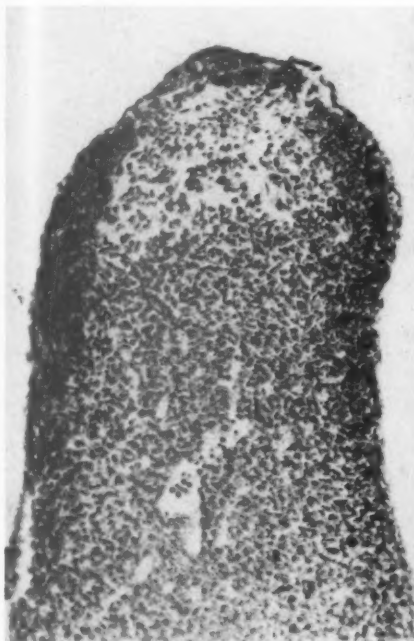
2

3



FIG. 3. Encephalocoele, skull and eye defects characteristic of irradiation on the 11th day. Hematoxylin and eosin stain. Low power.

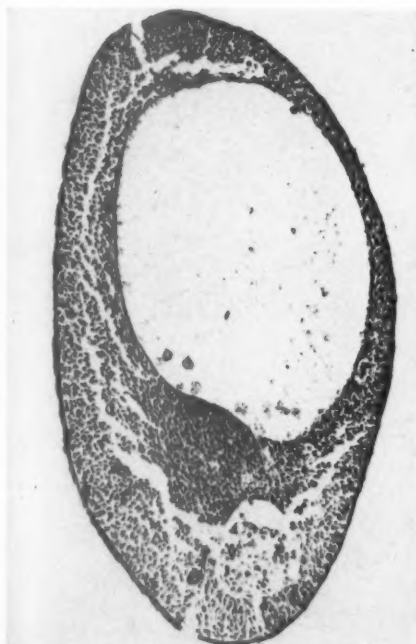
FIG. 4. Stepwise development of cystic defects in the feet, 4, 24, 48, and 72 hours after administration of 200 r. Four littermates are represented. Hematoxylin and eosin stain. $\times 175$, 175, 125, and low power, respectively.



4 Hours



24 Hours



48 Hours

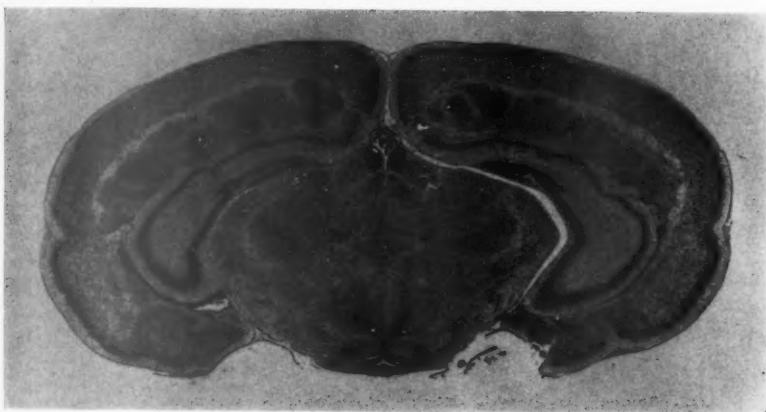


72 Hours

FIG. 5. The fate of rosettes is shown in the brains of three littermates 1, 10, and 14 days old. They were irradiated on the 13th day of gestation. Echt cresyl violet stain, low power.



1 Day



5

10 Days



14 Days

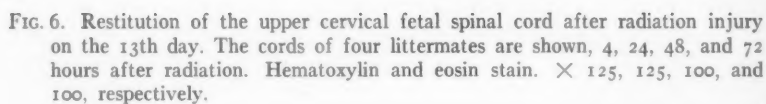


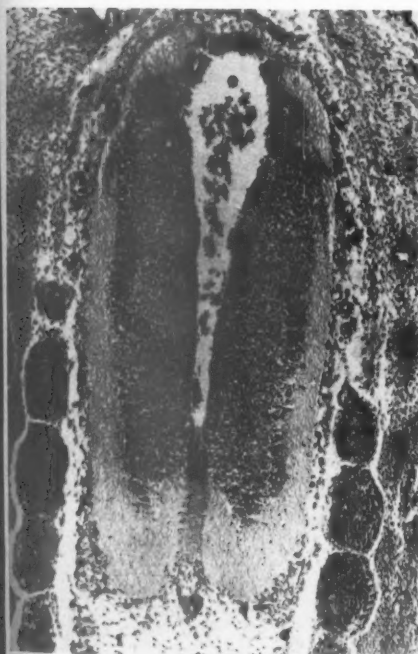
FIG. 6. Restitution of the upper cervical fetal spinal cord after radiation injury on the 13th day. The cords of four littermates are shown, 4, 24, 48, and 72 hours after radiation. Hematoxylin and eosin stain. \times 125, 125, 100, and 100, respectively.



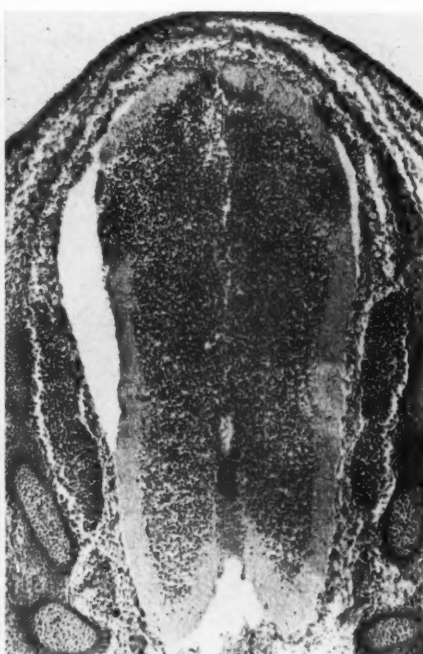
4 Hours



24 Hours



48 Hours



72 Hours



HETEROLOGOUS TRANSPLANTATION OF MALIGNANT TUMORS
TO THE ANTERIOR CHAMBER OF THE EYE IN GUINEA
PIGS TREATED WITH CORTISONE*

ABE TOWBIN, M.D.†

*From the Department of Pathology, Ohio State University,
College of Medicine, Columbus, Ohio*

The successful heterologous transplantation of tumor tissue, especially the cultivation of human neoplasms in laboratory animals, has long been a challenge to the experimental pathologist. In decades past it generally was conceded that the transplantation of human tumor tissue to animals of lower species was not feasible and that growth, if it occurred, was extremely rare. Despite these early unpromising views, a key to the problem ultimately was discovered. One of the most significant advances in experimental oncology in recent years has been the demonstration by Greene¹ that heterologous growth of human tumor tissue can be obtained by transplantation to the anterior chamber of the eye of the guinea pig.

The literature today contains reports from many laboratories describing the successful heterologous growth of human tumors of many types.²⁻⁵ However, with the available methods of heterologous transplantation, even the highly malignant human tumors show growth in but a relatively low percentage of cases. In recent years, in an effort to increase the proportion of successful transplantations, experimental studies have been pursued by many investigators to devise new methods of cultivating tumors heterologously.

A feasible method of growing malignant tumors routinely in laboratory animals would provide the pathologist with a promising avenue of approach in the study of tumor growth, differentiation, and spread.⁶ Clinically, it has been proposed that heterologous transplantation of human neoplasms may provide assistance diagnostically in distinguishing benign from malignant tumors.⁷ By using animals bearing tumor tissue transplanted from patients, it may ultimately be possible to test in advance the efficacy of irradiation, chemotherapy, or other treatment proposed for the patient.

Among the various techniques for growing tumors heterologously which have been described in recent years, two procedures have been

* This investigation was supported by research grant C1623 (C2) from the National Cancer Institute, of the National Institutes of Health, U. S. Public Health Service.

Presented at the Fifty-third Annual Meeting of the American Association of Pathologists and Bacteriologists, Cincinnati, Ohio, April 26, 1956.

Received for publication, June 29, 1956.

† Now at the Department of Pathology, College of Medicine, State University of New York at New York City, Brooklyn 2, N.Y.

of particular significance: (1) the anterior chamber method developed by Greene¹ and (2) the use of cortisone as an enhancing agent described by Toolan.⁸ In the present investigation these two procedures were linked. An appraisal of this combined cortisone-anterior-chamber method of hetero-transplantation of tumor tissue forms the basis of the present study.

The anterior chamber technique provides conditions uniquely favorable for the study of heterologous transplants. Prior to the development of this procedure by Greene,¹ attempts to grow tumor tissue in alien species met with little success. In devising a laboratory method of growing transplanted tissue, optimally, certain requisites must be met; the problem is not merely one of bringing about a tissue-culture-like growth of tumor cells; the transplant must be installed in an environment capable of supplying vascular and other stromal elements for the proliferating tumor cells. The tumor tissue which is ultimately elaborated should possess an organized histologic pattern with an incorporated stromal framework. These conditions are substantially attained by the method which utilizes the anterior chamber of the eye. A milieu favorable to the early development and growth of the transplant is available here; foreign body reaction tends to be less at this site than in other tissues. The anterior chamber method is particularly advantageous in that successive minute changes in the status of the transplant can be observed readily through the transparent cornea.

The administration of cortisone, in conjunction with the anterior chamber technique, was proposed in the present study as a means of providing a summation of conditions favorable for the cultivation of tumor tissue heterologously. Cortisone has been used in a wide range of transplantation procedures. Toolan⁸ has shown that when cortisone is administered to animals which have received human tumor tissue transplanted subcutaneously, the growth of the transplant is enhanced. Billingham, Krohn, and Medawar⁹ have shown that in the transplantation of skin from one rabbit to another, the grafts persist three to four times longer when cortisone is administered to the animals with the transplants.

The present investigation was carried on in two parts. In the first, using mouse tumor transplanted to the anterior chamber of the eye in cortisone-treated guinea pigs, an analysis was made of the mechanisms of action of cortisone in relation to the processes of heterologous transplantation. In the second part, the cortisone-anterior chamber method was applied in a study of heterologous transplantation of human tumors.

MECHANISMS OF ACTION OF CORTISONE IN THE HETEROLOGOUS TRANSPLANTATION OF TUMOR TISSUE

Past experiences with the anterior chamber method have shown that tumor tissue, after transplantation, is frequently destroyed by an overwhelming inflammatory reaction. This inflammation stems from two causes: (1) the incision and other trauma related to the transplantation; (2) the foreign body reaction in the tissues of the new host; this occurs mainly during the period of nidation,⁶ at the time when the tumor transplant is undergoing vascularization and organization prior to the stage of active growth. Cortisone has been shown to exert a suppressive effect upon the basic processes of inflammation; vascular permeability is reduced; exudation is diminished; migration of inflammatory cells is decreased; fibroplasia is inhibited; proliferation of granulation tissue is decreased.^{10,11} Accordingly, cortisone was employed in the present experiments in an effort to reduce the destructive inflammatory reaction associated with the process of heterologous transplantation.

In evaluating the effect of cortisone on heterologously transplanted tumor tissue in the anterior chamber, three reactions can be gauged: (1) inflammation, (2) the persistence of the transplant, and (3) the effect of cortisone on the growth of the transplant. A favorable effect exerted by cortisone would be evidenced by a reduction in the degree of inflammatory congestion and edema at the site of transplantation. Cortisone, if the action were favorable, would help maintain the transplant during the stage of nidation, in the period before the actual onset of growth; absorption of transplants would be inhibited. A favorable effect of cortisone on the growth of the transplant would be manifested by an acceleration in the onset of growth in the test animals as compared to the controls, by an increase in the rate of growth, and by a greater percentage of transplants showing growth in the treated animals. The following experiments were designed to assess these effects of cortisone in the heterologous transplantation of tumors.

EXPERIMENT IA: THE EFFECT OF CORTISONE ADMINISTERED LOCALLY ON THE POST-TRANSPLANTATION INFLAMMATORY REACTION

Material and Method

Mouse carcinoma of the breast type C₃HBA, obtained from the National Cancer Institute, Bethesda, Maryland, was employed in this study. This mouse tumor, which grows relatively well in the

guinea pig, provided an appropriate tissue for standardization studies. Tumor tissue was transplanted to the anterior chamber of the eye of the guinea pig using the technique outlined in previous studies.⁶ After anesthetizing the animal, the tumor transplant was introduced under sterile conditions into the anterior chamber of the eye with a no. 16 trocar through an incision at the limbus. Injection of cortisone in the eye subconjunctivally was the standard procedure in experiment IA. After the tumor tissue was installed in the eye, a deposit of cortisone (saline suspension containing 25 mg. of cortisone acetate* in 1 cc.) large enough to raise a 3 mm. bleb was injected under the corneal conjunctiva at a site opposite the incision. This technique was used initially to insure a lasting high local concentration of cortisone. In many instances after the transplantation procedure was completed, the injection of cortisone locally in the relatively small eye of the guinea pig proved difficult.

In this experiment, 48 guinea pigs were divided into groups of 16; the procedure was carried out three times in order that possible variations in environmental conditions, tumor tissue, and other factors might come under consideration. In each group of 16 animals with transplants, eight were treated with cortisone; the other eight were controls.

Inflammation in the eye which developed after transplantation was assessed according to changes of the following nature: the degree of corneal clouding and vascularization, the condition of the corneal incision, and the presence of iritis. The severity of inflammation was graded from 0° to 3°.

If the ocular structures were free of gross inflammatory changes, 0° (absence of inflammation), was the gradation applied. In such instances the transplant persisted intact, the cornea remained transparent, the edges of the incision were well approximated, and the iris was clear. Any one of the following changes was regarded as mild inflammation, 1°: mild corneal clouding, swelling of the ocular bulb, slight inflammation of the iris, or a small prolapse of the iris through the incision. If any one of the above changes was present in advanced degree, or if more than one inflammatory change of mild degree was present, 2° (moderate inflammation) was assessed. Severe inflammation, 3°, indicated a state of purulent ophthalmitis, with opaque cornea, ulcerated incision, and severe iritis.

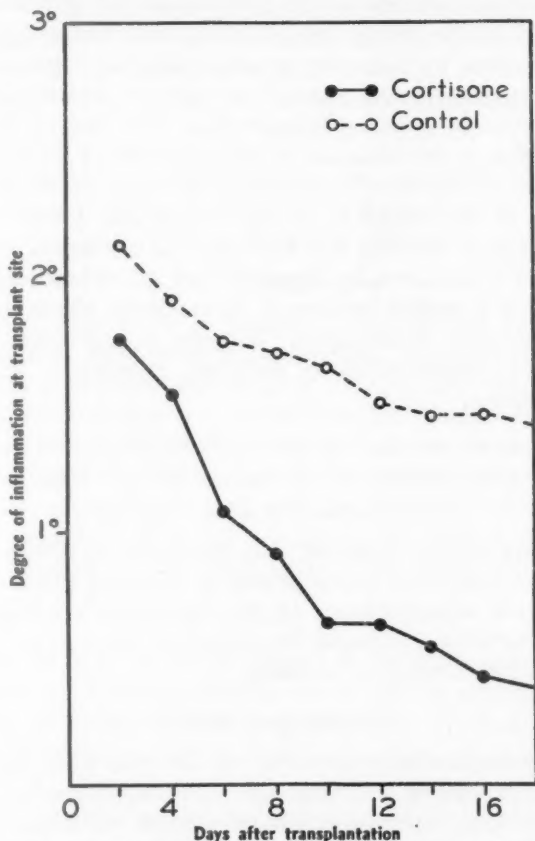
The animals were examined daily and the degree of ocular inflammation recorded. In selected instances the eyes containing transplants

* Obtained from Merck & Co., Inc., Rahway, New Jersey.

were harvested so that histologic changes could be studied and correlated with the gross changes in the transplant.

Results

The inflammatory changes which occurred in the three groups of animals, each comprising 16 guinea pigs, were comparable; accordingly, there were 24 animals with transplants which received cortisone and 24 served as controls. As evident in Text-figure 1, at the end of 2 days the degree of inflammation in the treated animals was generally



Text-figure 1. The effect of cortisone on post-transplantation inflammation. $C_{3}HBA$ carcinoma of the breast (mouse) was transplanted to the anterior chamber of the eye in 48 guinea pigs. The treated group of 24 animals received a single subconjunctival injection of cortisone after transplantation. Inflammatory reaction following transplantation was graded as follows: 0° = absence of inflammation, 1° = mild inflammation, 2° = moderate inflammation, and 3° = severe inflammation.

moderate, averaging 1.7° ; the degree of inflammation in the controls was similar, 2.2° . By the eighth day, a distinct difference was evident: in the treated animals the degree of inflammatory reaction was generally mild, averaging 0.9° ; in many animals the inflammatory process had completely subsided. In contrast, the control animals continued to show inflammatory changes of significant degree, averaging 1.7° . Often in the untreated animals destruction of the transplanted tumor tissue occurred as a consequence of heavy ocular inflammation.

The effect of cortisone on the persistence and growth of tumor tissue could not be clearly defined in this experiment. The C₃HBA mouse carcinoma, by its nature, grows rapidly in the guinea pig eye. The single subconjunctival injection of cortisone, although exerting a favorable effect by reducing inflammation, could not be shown definitely to influence the initiation or rate of growth of the transplanted tumor tissue. To appraise the effects of cortisone on the persistence and growth of the transplant, it was evident that a more prolonged administration of the drug was required. An evaluation of the prolonged effect of cortisone, by repeated local subconjunctival administration, was not feasible because of the traumatic effects of repeated corneal injections. Consequently, in order to determine the effect of prolonged administration of cortisone, experiments IB and IC were undertaken.

EXPERIMENT IB: THE EFFECT OF PROLONGED PARENTERAL
ADMINISTRATION OF CORTISONE ON POST-
TRANSPLANTATION INFLAMMATION

Preliminary studies indicated that parenteral injections of cortisone, intramuscularly or intraperitoneally, produced effects similar to subconjunctival administration. In this experiment, a standardization study was carried out to define the optimal dosage pattern for intraperitoneal administration of cortisone.

Material and Method

The procedure for transplantation was the same as in the previous experiment. A total of 80 guinea pigs with transplants were studied. In order to bring under consideration possible variations in experimental conditions, the animals were studied in groups of eight. The following procedure was repeated ten times: Cortisone was injected intraperitoneally in a daily dosage of 10 mg. per kg. of body weight. In each group of eight guinea pigs, two animals received cortisone

for 8 days prior to transplantation. Two of the animals received cortisone for 16 days after transplantation. Two animals in each group received a daily injection of cortisone for 8 days before transplantation and for 16 days thereafter. Two of the guinea pigs with transplants served as untreated controls.

Results

Optimal effects in controlling inflammation were evident in the animals treated with cortisone for 8 days before and 16 days after transplantation. This pattern of administration was adopted in subsequent experimental studies. The experiment also provided preliminary evidence that cortisone, administered over an extended period, enhanced the persistence and growth of anterior chamber transplants of mouse tumor.

EXPERIMENT IC: THE EFFECT OF CORTISONE ON PERSISTENCE AND GROWTH OF HETEROLOGOUS TRANSPLANTS OF MOUSE TUMOR

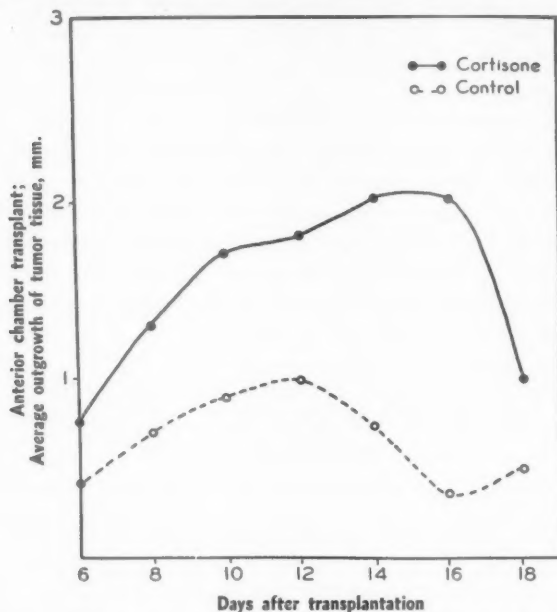
Material and Method

Transplants were made in groups of eight guinea pigs by the anterior chamber method as in the previous experiments. In each group, four animals were treated with cortisone and four served as controls. This procedure was carried out ten times. Accordingly, a total of 40 test animals and a like number of controls were studied. Cortisone was administered in accordance with the optimal pattern defined in experiment IB: 10 mg. per kg. of body weight intraperitoneally for 8 days before and 16 days after transplantation. The transplants were studied in detail with a magnifying lens to detect early evidence of growth.

Results

The effect of cortisone on the growth of the transplant is evident in Text-figure 2. Onset of growth was earlier in cortisone treated animals: by the sixth day after transplantation, 17 of the cortisone treated animals showed tumor growth as compared to ten of the control animals. Transplants in the cortisone treated group grew, on an average, twice the size of the control transplants. Many of the transplants reached a diameter of 6 mm. or more; in the control animals, such growth was infrequent. The duration of tumor growth was greater in the cortisone treated animals. In the control group, 20 per cent of the transplants failed to persist and grow, and were

absorbed at an early period whereas in the cortisone treated animals, 10 per cent of the transplants failed to show growth.



Text-figure 2. The effect of cortisone on the growth of heterologously transplanted tumor tissue. C₃HBA carcinoma of the breast (mouse) was transplanted to the anterior chamber of the eye of the guinea pig. Cortisone was administered intraperitoneally.

HETEROLOGOUS TRANSPLANTATION OF HUMAN TUMORS USING THE CORTISONE-ANTERIOR-CHAMBER METHOD

Certain tumors of animals grow readily when transplanted heterologously⁵: most malignant tumors of the mouse, and many tumors of the rat and rabbit, can be grown in the guinea pig without difficulty. As the degree of species difference between the donor and recipient animals widens, however, heterotransplantability of neoplasms decreases. This factor becomes immediately evident when tumors from a series of animals of ascending order are transplanted to the guinea pig. Successful transplantation, although easily obtained with mouse tumors, occurs with decreasing percentage when tumors from dog, horse, and man are used. In a previous study in this laboratory,⁵ 100 clinically malignant human neoplasms were transplanted to the guinea pig: active growth of transplants was evident with only two of the tumors; transplants from nine of the tumors persisted for extended

periods but failed to show active proliferation; transplants of 89 of the tumors regressed at an early stage.

The results noted above in the first part of the present investigation, in which the cortisone-anterior-chamber method of heterologous transplantation was applied to mouse tumor, provided evidence that this method affords a means of enhancing the growth of heterologous tumor transplants. Consequently, a study was undertaken in which this procedure was applied to the heterologous transplantation of human tumors, in an effort to obtain a greater yield of successful transplantations.

Material and Method

Ninety-four human tumors, considered to be unequivocally malignant, were transplanted to the anterior chamber of the eye in cortisone treated guinea pigs. The diagnosis of malignancy was determined by the clinical behavior of the neoplasm, the histologic pattern of the tumor, the gross local invasiveness, and, in most cases, by the presence of metastases. Most tumors were obtained from specimens taken for biopsy in the course of surgical procedures, but a few were obtained under aseptic conditions at necropsy shortly after death. A portion of each specimen was studied histologically. An effort was made to select well preserved, cellular tumor tissue for transplantation. With each tumor, eight animals received transplants, four of which received cortisone and four remained as controls. Cortisone was administered to the test animals in accordance with the optimal dosage pattern determined earlier in the present study. Test animals received 10 mg. of cortisone per kg. of body weight usually for 8 days prior to transplantation and for 16 days thereafter. The number of days that the animals received cortisone before transplantation varied to some extent depending upon the availability of specimens and other exigencies. Animals with growing transplants were maintained until blanching or other evidence of regression of the tumor tissue was apparent in either the control or treated animals. The histologic pattern of the tumor tissue from the patient was correlated with that obtained from the cortisone treated animal and from the controls.

Results

The results of this experiment are indicated in Table I, in which growth is recorded as 1 plus when enlargement of the transplant up to 5 mm. occurred and as 2 plus when there was growth of greater extent.

Of the 94 human neoplasms studied, tissue from 14 tumors, 14.9

TABLE I
*Successful Heterologous Transplants of Human Tumors**

Acc. no.	Type of tumor	Source of transplant	Corticone treated guinea pigs		Control guinea pigs	
			Transplant growth†	Onset of growth	Transplant growth†	Onset of growth
13S	Bronchogenic carcinoma	Lung	+	23rd day		
18W	Bronchogenic carcinoma	Lung	+	21st day		
24V	Bronchogenic carcinoma	Lymph node metastasis	+	39th day	+	56th day
28F	Bronchogenic carcinoma	Lung	+	64th day		
16P	Spongiblastoma multiforme	Brain	+	42nd day	+	58th day
21K	Ependymoma	Brain			++	97th day
27K	Spongiblastoma multiforme	Brain	+	18th day		
23U	Carcinoma of breast	Lymph node metastasis	+	34th day		
13U	Malignant melanoma of skin	Lymph node metastasis	+	28th day		
22A	Craniopharyngioma	Sphenoid bone	+	59th day		
31U	Carcinoma of testis	Testis	+	20th day		
27T	Osteosarcoma	Bone	++	91st day	+	184th day
13Q	Fibrosarcoma of thigh	Lymph node metastasis	+	17th day		
15F	Fibrosarcoma	Mediastinum	++	56th day		143rd day
29Q	Fibrosarcoma	Vulva	++	10th day		

* Of the 94 tumors studied, growth of transplants failed to occur in treated or control animals in the following 79 cases: 18 bronchogenic carcinomas, 14 gliogenous tumors of the central nervous system, 9 carcinomas of the breast, 5 carcinomas of the renal cortex, 4 carcinomas of the colon, 3 carcinomas of the salivary gland, 3 carcinomas of the thyroid gland, 2 carcinomas each of tongue, liver, pharynx, testis, 7 soft tissue sarcomas, and 1 laryngeal carcinoma, prostatic carcinoma, carcinoma of uterine cervix, malignant melanoma of skin, meningeal sarcoma, thymoma, adrenal neuroblastoma, and osteogenic sarcoma of bone.

† Results are recorded as 1 plus when enlargement of the transplant up to 5 mm. occurred and as 2 plus when there was growth of greater extent.

per cent, grew in the anterior chamber of the eye of cortisone treated guinea pigs. In the control animals which did not receive cortisone, growth of transplants occurred in five, 5.3 per cent. The difference between 14.9 per cent and 5.3 per cent is a measure of the effect of cortisone in augmenting transplantability.

In addition to enhancing the growth of the transplanted tumors, other effects of cortisone were evident in this experiment. As in previous studies using mouse tumors, the inflammatory reaction at the site of transplantation was less intense in the cortisone treated guinea pigs than in the control animals. Whether growth ensued or not, the transplanted tumor tissue tended to endure and remain visible longer in the cortisone treated animals than in the controls. In the former, tumor transplants which failed to grow remained visible in the anterior chamber for periods averaging 41 days; in the latter, 26 days. This greater persistence of the transplants in the treated guinea pigs was evident with 78 per cent of the tumors studied.

The transplants of ten tumors grew in the cortisone treated animals only; those of four tumors grew in both the cortisone treated animals and the controls; one tumor, an ependymoma, grew in a control animal but not in the guinea pigs which received cortisone. Of the various types of malignant tumors transplanted, bronchogenic carcinomas grew in a slightly higher percentage of cases than other neoplasms. Four of the 22 bronchogenic carcinomas, 18 per cent, grew in cortisone treated guinea pigs.

The effect of cortisone in augmenting the growth of transplants was distinctly evident in the four instances in which transplants grew in both the cortisone treated animals and in the controls. In each case the onset of growth occurred earlier and the volume of tumor tissue was greater in the cortisone treated animals (Table I). For example, with the transplants of tumor no. 16P, onset of growth was observed in the cortisone treated animal on the 42nd day; in the control, 16 days later. On the 72nd day, the tumor transplant in the cortisone treated animal was 6 mm. in diameter while in the control it was 4 mm. At that time the tumor tissue in the eye of the control guinea pig appeared pale and slightly contracted and it was harvested together with that of the cortisone treated animal. Histologically, the material from the latter proved to be a close replica of the original tumor taken from the patient at operation. Figure 1 is a section of an eye with tumor tissue showing the characteristic pleomorphic pattern of spongioblastoma multiforme growing in the anterior chamber near the iris. The tumor tissue from the control animal was similar, but showed early necrosis. Likewise with other

tumors which grew, the identity of the histologic pattern of the original tumor tissue remained well preserved in the transplanted neoplastic tissue. In tumor no. 15F, a fibrosarcoma, the transplant became attached to the inner aspect of the cornea and grew out into the anterior chamber. The histologic characteristics of fibrosarcoma were readily evident in the tumor tissue harvested from the cortisone treated guinea pig (Fig. 2). Figure 3 shows a section of tumor tissue, bronchogenic carcinoma, no. 24V, harvested from a cortisone treated guinea pig. Figure 4 is a section of craniopharyngioma, tumor no. 22A, from a cortisone treated animal.

DISCUSSION

The 94 selected human tumors in the present study included a relatively large number of bronchogenic carcinomas and malignant astrocytomas. Observations in previous studies had suggested that these two forms of malignant growths, as compared to other human neoplasms, have a high natural propensity for growth in the anterior chamber of the guinea pig eye. Therefore, in the present study, these tumors were included in relative excess intentionally. It should be emphasized that the present study was designed primarily to test the effect of cortisone with the anterior chamber technique, and was not intended as an appraisal of the transplantability of a cross-sectional incidence of human neoplasms. Accordingly, the results in the present study cannot be compared directly with previous investigative findings, in which heterologous growth occurred with two of 100 successive human malignant tumors.⁶ The difference between the percentage of successful transplants observed in the earlier study, 2 per cent, and that in the untreated control guinea pigs in the present study, 5.3 per cent, is in all likelihood not due to a fortuitous biologic circumstance, nor is it attributable to an added adeptness in applying the transplantation procedure, but may be related to the selected nature of the tumors in the later study.

In the cultivation of malignant tumors in laboratory animals, transplantation sites other than the anterior chamber have been tested. Resolute attempts have shown that tumors in certain instances can be grown heterologously in the brain, the testis, and other locations. Standardization studies have been carried out in this laboratory comparing the anterior chamber of the eye as a site of transplantation with the brain and other organs. With transplants deposited blindly in the brain and other deep locations, tumor growth of substantial degree may occur and escape detection. Using the anterior chamber

as the site of transplantation, changes in the transplant are readily visible through the transparent cornea. The condition of the tumor transplant can be observed from day to day and the tissue in the anterior chamber may be harvested at the time of maximal growth. In this laboratory, the anterior chamber of the eye has proved to be the site most suitable for studies of heterologous transplantation of human tumors. The present study indicates that the administration of cortisone increased the heterologous transplantability of human tumors to the anterior chamber of guinea pigs three-fold.

SUMMARY

Various methods of transplanting tumor tissue heterologously have been devised and efforts have been made to increase the percentage of successful transplantations. Two experimental developments, the anterior chamber technique and the administration of cortisone to the host, are of salient importance. In the present investigation these two procedures were combined. Using mouse tumors transplanted to guinea pigs, studies were made of the effects of cortisone on the processes of heterologous transplantation. Cortisone tended to suppress the post-transplantation inflammatory reaction, to increase the persistence of tumor transplants, and to enhance the growth of the transplanted tumor tissue.

The cortisone-anterior-chamber method was then applied in a study of heterologous transplantation of human tumors. Growth of transplants was obtained with 14 of 94 human tumors transplanted to the anterior chamber of the eye of cortisone treated guinea pigs. Five of the 14 tumors also grew in untreated control animals. Tumor growth generally occurred earlier and was more extensive in the animals receiving cortisone.

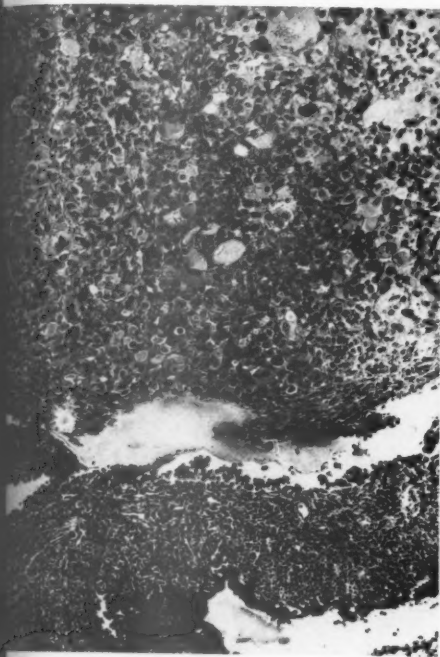
REFERENCES

1. Greene, H. S. N. Heterologous transplantation of mammalian tumors. II. The transfer of human tumors to alien species. *J. Exper. Med.* 1941, 73, 475-486.
2. Jones, E. G.; Hale, C. W., and Dambach, B. Heterologous transplantation of human malignant ovarian tumor. Report of successful growth of human papillary cystadenocarcinoma in anterior chamber of eye of guinea pig. *Am. J. Obst. & Gynec.*, 1946, 51, 893-896.
3. Schilling, J. A.; Snell, A. C., Jr., and Favata, B. V. Heterologous ocular transplantation as a practical test for cancer. *Cancer*, 1949, 2, 480-490.
4. Morris, D. S.; McDonald, J. R., and Mann, F. C. Intra-ocular transplantation of heterologous tissues. *Cancer Research*, 1950, 10, 36-48.
5. Towbin, A. The heterologous transplantation of human tumors. *Cancer Research*, 1951, 11, 716-722.

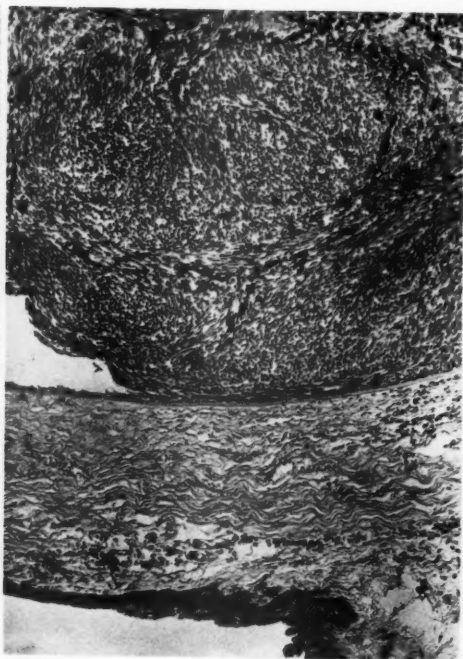
6. Towbin, A. A study of tumor tissue after transplantation. Histologic changes observed in anterior-chamber transplants of heterologous tumor tissue. *A. M. A. Arch. Path.*, 1951, 52, 199-209.
7. Greene, H. S. N. Identification of malignant tissues. *J. A. M. A.*, 1948, 137, 1364-1366.
8. Toolan, H. W. Growth of human tumors in cortisone-treated laboratory animals: the possibility of obtaining permanently transplantable human tumors. *Cancer Research*, 1953, 13, 389-394.
9. Billingham, R. E.; Krohn, P. L., and Medawar, P. B. Effect of cortisone on survival of skin homografts in rabbits. *Brit. M. J.*, 1951, 1, 1157-1163.
10. Ragan, C.; Howes, E. L.; Plotz, C. M.; Meyer, K., and Blunt, J. W. Effect of cortisone on production of granulation tissue in the rabbit. *Proc. Soc. Exper. Biol. & Med.*, 1949, 72, 718-721.
11. Spain, D. M., and Molomut, N. Effect of cortisone on inflammation in mice. Minimum dose required to affect eosinophil count, inflammation, wound healing and size of spleen. *Am. J. Clin. Path.*, 1952, 22, 944-947.

LEGENDS FOR FIGURES

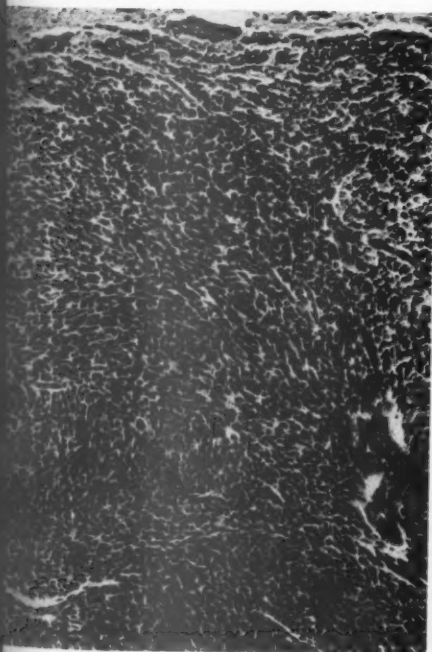
- FIG. 1. Growth of human tumor, no. 16P, in the anterior chamber of the eye of a cortisone treated guinea pig. The tumor tissue, growing near the iris, has the pattern characteristic of spongioblastoma multiforme. Hematoxylin and eosin stain. $\times 170$.
- FIG. 2. Growth of human tumor, no. 15F, in the anterior chamber of the eye of a cortisone treated guinea pig. The tumor, a fibrosarcoma, is attached to the inner aspect of the cornea. Hematoxylin and eosin stain. $\times 80$.
- FIG. 3. Bronchogenic carcinoma, no. 24V. Tumor tissue was harvested from a cortisone treated guinea pig. Hematoxylin and eosin stain. $\times 120$.
- FIG. 4. Craniopharyngioma, no. 22A. Tumor tissue was harvested from a cortisone treated guinea pig. Hematoxylin and eosin stain. $\times 170$.



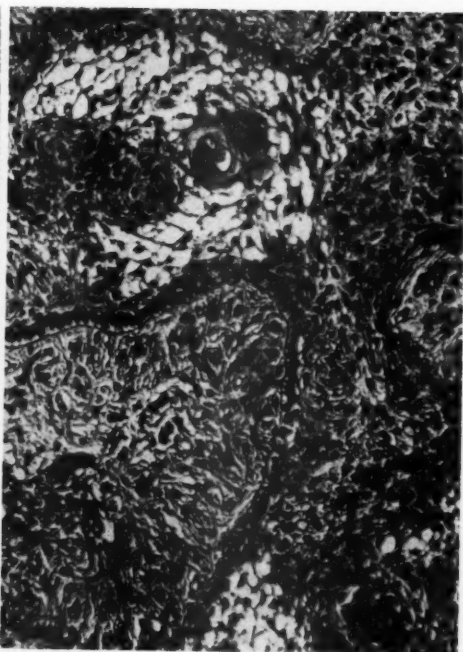
1



2



3



4

CARCINOMA OF THE PANCREAS*

I. A CLINICAL AND PATHOLOGIC STUDY OF 609 NECROPSIED CASES

II. THE RELATION OF CARCINOMA OF THE PANCREAS TO DIABETES MELLITUS

E. T. BELL, M.D.

From the Department of Pathology, University of Minnesota, Minneapolis 14, Minn.

This study includes all of the cases of carcinoma of the pancreas in the necropsy records of the Department of Pathology at the University of Minnesota from January 1, 1911, through December 31, 1954. The records comprise 46,847 males and 26,340 females (Table I).

During the period 1911 to 1954, protocols and gross specimens of practically all necropsies performed in the Twin City area (Minneapolis and St. Paul) were filed in the Department of Pathology. Contributors included all the private hospitals and the large municipal hospitals of Minneapolis and St. Paul, the University Hospitals, the Coroner's service of Hennepin County, and, in recent years, the Veterans Administration Hospital at Minneapolis. During the last decade the number of necropsies was much greater than in earlier years. During the 4-year period 1949 to 1952, the number of necropsies was equal to about one third of the deaths in males and about one fourth of the deaths in females in the Twin City area. This is a large, widely distributed sample, and at least in recent years it is probably fairly representative of the diseases in this locality. Certainly such a large necropsy sample gives a more accurate estimate of the incidence of carcinoma of the pancreas than that based upon death certificates, since the clinical diagnosis of this neoplasm is often uncertain.

Only primary carcinomas of the pancreas are tabulated. Carcinomas arising in the papilla of Vater or the extrapancreatic portions of the bile ducts are excluded. Also omitted are a few cases in which the pathologist was unable to exclude a primary source outside the pancreas. The carcinoma was an accidental necropsy finding in two cases; in all others it was the major cause of death.

* Aided by a grant from the U.S. Public Health Service.

Received for publication, June 30, 1956.

I. A CLINICAL AND PATHOLOGIC STUDY OF 609 NECROPSIED CASES

Frequency

Carcinoma of the pancreas was found in 0.91 per cent of all the males, and in 1.23 per cent of those over 40 years of age (Table I). It was somewhat less frequent in females, occurring in 0.70 per cent of the entire group and in 1.10 per cent of those over 40 years of age (Table I).

Age Incidence. In males, 97 per cent of carcinomas of the pancreas occurred in subjects over 40 years of age. The youngest male was 27 years old. The tumor increased in frequency with advancing age, reaching a maximum in the eighth decade (Table I). In females, 98 per cent of the tumors occurred in subjects over 40 years of age, but there was no increase in frequency with advancing age as in males. The youngest female was 27 years old (Table I). Several

TABLE I
Incidence of Carcinoma of the Pancreas in Necropsies

Decades	Males			Females		
	Total necropsies	Carcinoma of pancreas		Total necropsies	Carcinoma of pancreas	
		No.	%		No.	%
0- 10 yrs.	6,815	0	0.00	4,793	0	0.00
10- 20 yrs.	1,173	0	0.00	962	0	0.00
20- 30 yrs.	2,125	1	0.05	1,843	1	0.05
30- 40 yrs.	3,367	14	0.42	2,211	2	0.09
40- 50 yrs.	5,616	40	0.71	2,950	12	0.41
50- 60 yrs.	8,590	82	0.95	3,748	48	1.28
60- 70 yrs.	9,441	140	1.48	4,246	52	1.22
70- 80 yrs.	6,969	120	1.72	3,786	52	1.37
80-100 yrs.	2,751	27	0.98	1,801	18	1.00
Total	46,847	424	0.91	26,340	185	0.70
40-100 yrs.	33,367	409	1.23	16,531	182	1.10

reports mention a case in the third decade. Corner¹ described a primary carcinoma of the pancreas in an infant 7 months old, and stated that he found reports of five cases in children. Grant and Perceval² reported a carcinoma of the pancreas in a girl 16 years of age. The disease is evidently rare before the age of 30 years.

Sex Incidence. In our necropsy records there were 424 males and 185 females, but there were about twice as many adult males as adult females in the necropsy population. When computed on a percentage basis, the preponderance in males was only about 15 per cent.

In necropsy statistics from other sources there are usually over twice as many males as females, but the authors do not give the proportion of males to females in the necropsy population from which their statistics are derived. In clinical reports, males usually predominate in a ratio of about 2 to 1; but the diagnosis is commonly based on necropsy findings and is subject to the same error.

The Increase in Carcinoma of the Pancreas. Since over 85 per cent of all cancer deaths and over 97 per cent of all deaths from carcinoma of the pancreas occur after the age of 40 years, a more accurate estimate is obtained by tabulating the subjects over 40 years of age as in Table II. The incidence of carcinoma of the pancreas in males over 40 years old at death increased from 0.98 per cent in the 35-year period, 1911 to 1944, to 1.57 per cent in the 10-year period, 1945 to 1954. This represents an increase of 60 per cent. During these corresponding periods, deaths from all malignant neoplasms in males

TABLE II
Incidence of Fatal Neoplasms and Carcinoma of Pancreas for Two Periods

Males over 40 years of age					
Period	Total necropsies	Fatal neoplasms		Carcinoma of pancreas	
		No. of cases	Percentage of total necropsies	Percentage of total necropsies	Percentage of fatal neoplasms
1911-1944	19,526	3,827	19.60	0.98	5.00
1945-1954	13,841	3,651	26.38	1.57	6.00
1911-1954	33,367	7,478	22.41	1.23	5.49

Females over 40 years of age					
Period	Total necropsies	No. of cases	Percentage of total necropsies	Carcinoma of pancreas	
				Percentage of total necropsies	Percentage of fatal neoplasms
1911-1944	9,139	2,211	24.20	1.03	4.25
1945-1954	7,392	2,013	27.23	1.19	4.37
1911-1954	16,531	4,224	25.55	1.10	4.31

over 40 years old advanced from 19.60 per cent to 26.38 per cent, an increase of about 35 per cent (Table II). This relatively greater increase of carcinoma of the pancreas is indicated by the fact that it comprised about 5 per cent of the fatal neoplasms in the period 1911 to 1944 and 6 per cent during the period 1945 to 1954 (Table II). The increased frequency of fatal neoplasms in males in recent years is due to the decreased number of deaths from infectious diseases and the increased number of very old men in the community. Carcinoma of the pancreas has its maximum incidence in males in the eighth decade, and two thirds of its victims are over 60 years of age.

It is not surprising, therefore, that it now comprises a larger percentage of fatal neoplasms than it did formerly.

In females over 40 years of age fatal neoplasms have increased only 12.5 per cent in the two periods mentioned (Table II), about one third of the increase noted in males. This phenomenon is probably due to the fact that cancer is more common in women than in men under 50 years of age, and less frequent in women than in men thereafter. The increasing age of the population, therefore, has less effect on the incidence of cancer in females. Carcinoma of the pancreas has increased about the same as other fatal neoplasms, and it comprises about the same percentage of fatal neoplasms in the two periods (Table II).

Macroscopic Types of Carcinoma of the Pancreas

On the basis of the gross structure, four types of carcinoma may be recognized: carcinoma of the head, of the body, of the tail, and a diffuse form. The separation of the several types is somewhat arbitrary. In this discussion, carcinoma of the head includes all cases with massive involvement of the head with or without extension into the rest of the gland. It also includes all small tumors restricted to the head. Carcinoma of the body refers to cases in which the head is free of gross tumor and the growth occupies the body alone or the body and the tail. Carcinoma of the tail includes all cases in which the only gross tumor is in the tail. The diffuse type refers to those with multiple nodules throughout the gland without complete replacement of any segment. It is obvious that there may be gross invasion or compression of the common bile duct in carcinoma of the head and in diffuse carcinoma; but in carcinoma of the body or tail, obstruction of the duct can be accomplished only by extension or metastases. One would anticipate, therefore, that jaundice would be absent or delayed in carcinomas of the body or tail, and that small tumor masses in the head would not necessarily cause jaundice. On the basis of the above definitions, the 609 carcinomas were grouped as follows: Carcinoma of the head, 360 cases, 59.1 per cent; of the body, 111 cases, 18.2 per cent; of the tail, 45 cases, 7.4 per cent; and the diffuse type, 93 cases, 15.3 per cent. The reports of various observers are in agreement that about two thirds of the cancers involve the head. Minor discrepancies are due to differences in the number classified as diffuse.

The four gross anatomical types of carcinoma of the pancreas will now be analyzed with respect to their clinical features. The over-all

ratio of males to females is about 7 to 3, and this ratio obtains roughly for the four groups. There is no evident sex predisposition to any type.

Duration of Symptoms

The duration of symptoms is by no means an accurate measure of the length of time a neoplasm has been present. Symptoms commonly do not develop until the tumor obstructs the common bile duct or extends beyond the pancreas by infiltration or metastases. The occasional finding of an asymptomatic carcinoma at necropsy indicates that there is an asymptomatic early stage, but we do not know its duration. It appears highly probable that many cancers are present a long time before the appearance of pain or jaundice. This consideration should be kept in mind in determining the relation between pancreatic carcinoma and diabetes mellitus.

Our 547 cases in which the duration of symptoms was known are shown in Table III. Broadbent and Kerman,³ as well as Silver and Lubliner,⁴ found a much longer duration of symptoms in carcinomas of the body and tail than in carcinomas of the head, but Duff⁵ found about the same average duration in the two groups. Our data (Table

TABLE III
*Duration of Clinical Symptoms for the Four Anatomical Types
of Carcinoma of the Pancreas (547 Cases)*

Duration of symptoms	Head	Body	Tail	Diffuse
With known duration, 547	331	95	38	83
0 to 3 months	77	18	10	21
3 to 6 months	86	26	10	21
6 to 12 months	108	35	15	24
1 to 2 years	42	10	1	13
2 to 5 years	18	6	2	4
Unknown duration	29	16	7	10

III) show no significant differences in the duration of symptoms in the four groups. The duration varied from 1 month to 5 years. About one half of the patients died within 6 months after the appearance of the first symptom; and 17.5 per cent survived over 1 year. In those who survived over 2 years there may be inaccuracies in determining the time of onset, since some cases were complicated by the presence of gallstones.

Initial Symptoms

We are concerned here only with the initial or presenting symptoms and not with the late manifestations of the neoplasm. The frequency with which the various symptoms and signs appeared at the onset

of the illness is shown in Table IV, with respect to the four gross anatomical types. More than one symptom was usually present initially.

TABLE IV

Initial Symptoms in Carcinoma of the Pancreas with Respect to the Four Anatomical Types

	Anatomical type				Total	
	Head	Body	Tail	Diffuse	No.	Per cent
No. of cases analyzed	333	107	43	90	573	100.0
Abdominal pain or distress	183	61	22	55	321	56.0
Jaundice	142	2	0	13	157	27.4
Nausea and vomiting	49	9	3	11	72	12.6
Anorexia	47	13	8	7	75	13.1
Weakness	42	9	5	3	59	10.3
Loss of weight	19	10	6	11	46	8.0
Constipation	13	4	2	6	25	4.4
Diarrhea	23	8	1	3	35	6.1
Backache	12	10	2	1	25	4.4
Belching	7	1	1	2	11	1.9
Fever	4	0	1	1	6	1.0
Enlarged abdomen	6	10	3	6	25	4.4
Pulmonary metastases	4	2	1	2	9	1.6
Metastases to bone	0	3	0	3	6	1.0
Metastases to brain or spinal cord	0	1	3	2	6	1.0
Metastases to peripheral lymph nodes	4	1	0	1	6	1.0
Thrombophlebitis	3	1	2	3	9	1.6

TABLE V

The Incidence of Jaundice in the Four Anatomical Types of Carcinoma of the Pancreas

Site	No. of cases analyzed	Jaundice present at onset		Jaundice appeared later		Painless jaundice		No jaundice	
		No.	%	No.	%	No.	%	No.	%
Head	339	142	41.9	137	40.4	78	23.0	60	17.7
Body	109	2		19		0		88	80.7
Tail	45	0		7		0		38	84.4
Diffuse	92	13		38		2		41	44.7
Total	585	157	26.8	201	34.4	80	13.7	227	38.8

Abdominal Pain or Discomfort. This is the most frequent initial symptom in all four types of pancreatic carcinoma. It varies in intensity from a feeling of abdominal discomfort to a severe boring pain. It is most often located in the epigastric region but may be

felt in either hypochondriac region or in the lower abdomen. It is often described as deeply placed. In our 573 cases with adequate history, abdominal pain was an initial symptom in 56 per cent, and its frequency was about the same in the four anatomical types. In the later stages of the disease this symptom is present in a much higher percentage of the patients. A number of observers reported abdominal pain as an initial symptom in 50 per cent or more of their patients (Duff,⁵ Country and Foulk,⁶ Brown *et al.*,⁷ Smith and Albright,⁸ Berk,⁹ Sanders and McBurney¹⁰).

Jaundice. The occurrence of jaundice is shown in detail in Table V. It was present initially in 41.9 per cent of carcinomas of the head, and developed later in an additional 40.4 per cent. In one sixth of the carcinomas of the head, jaundice never developed. In 78 cases (23 per cent) there was a painless jaundice at the onset of the disease. In 109 cases of carcinoma of the body of the pancreas, jaundice was present initially in two and developed later in 19. In 80.7 per cent there was no jaundice at any time. No case of carcinoma of the tail showed an initial jaundice and in 84.4 per cent jaundice never appeared. In the diffuse type, 13 of 92 cases showed an initial jaundice and 38 developed jaundice later. In the entire group of 585 cases, jaundice was an initial symptom in 26.8 per cent and appeared later in 34.4 per cent. There was no jaundice at any time in 38.8 per cent. Painless jaundice was an initial symptom in 23 per cent of carcinomas of the head and in 13.7 per cent of the entire group. In collected statistics Berk⁹ found painless jaundice to be an initial symptom in 17.4 per cent of pancreatic carcinomas. An onset of jaundice with pain is just as frequent as painless jaundice.

In carcinomas of the head, jaundice is clearly due to invasion or compression of the common bile duct. Those cases without jaundice had smaller tumors that did not destroy the entire head of the gland. In a few cases of carcinoma of the body or tail, jaundice appeared late in the disease without gross involvement of the head. Kaplan and Angrist¹¹ have shown that this jaundice is also obstructive in origin, being due to small metastases about the bile duct. It does not seem to be due to destruction of the liver, since there are a number of cases in this series with massive metastases in the liver without jaundice.

Other Initial Symptoms. Anorexia, nausea and vomiting were frequent early symptoms. Weakness and loss of weight often occurred several months before any other symptoms were noted. Constipation was noted as an initial symptom in 25 cases (4.4 per cent). This low incidence is probably due to incomplete clinical records since Thomp-

son and Rodgers¹² found constipation at the onset in 36 per cent, Brown *et al.*⁷ in 45 per cent, and Berk⁹ in 38.7 per cent.

Diarrhea was an outstanding initial symptom in 35 patients (6.1 per cent). Thompson and Rodgers¹² found an initial diarrhea in 15 per cent; Dashiell and Palmer,¹³ in 20 per cent; and Berk,⁹ in 10.8 per cent. The stool was usually described as watery and foul-smelling, but occasionally it was bulky and fatty and considered as typical of steatorrhea. Fatty stools are presumably due to destruction of the acinar tissue of the pancreas. Cantor and Haking¹⁴ reported a patient with intractable watery diarrhea who presented the roentgen pattern in the intestines of non-tropical sprue.

Fever was an initial symptom in only 6 cases (1 per cent). The patient first consulted a physician because of enlargement of the abdomen in 25 instances (4.4 per cent). The enlargement was due to ascites, a large liver, or to the pancreatic carcinoma.

There were 27 patients in whom the initial symptoms were due to distant metastases (4.6 per cent). In nine patients the presenting symptoms were cough, dyspnea, and hemoptysis resulting from extensive pulmonary metastases. In six subjects severe bone pain called attention to metastases which were then demonstrated by roentgen examination. The primary sites of the neoplasm were not known at that time. In five subjects the first sign was enlargement of cervical lymph nodes; and in one patient enlarged inguinal nodes, which proved to be metastatic carcinoma on biopsy. In six cases the disease began with signs of a tumor of the brain or spinal cord, which later proved to be metastatic from the pancreas.

Venous Thrombosis

In recent years a number of papers have been published which emphasize the frequency of venous thrombosis in association with pancreatic carcinoma. It is stated that venous thrombosis occurs more frequently with pancreatic than with other carcinomas, and some writers believe that venous thrombosis suggests carcinoma of the body or tail of the pancreas.

Phlebothrombosis. It is necessary to distinguish terminal phlebothrombosis from thrombophlebitis. Phlebothrombosis of the veins of the lower extremities is a common terminal complication of many diseases, notably cardiac failure and cancer. Such thrombi may be found in the legs in over 50 per cent of middle-aged or elderly subjects. They are attributed to circulatory failure and are not specific for any disease. They may cause edema of the lower extremities.

Bilateral edema of the lower extremities was noted in about 30 per cent of our cases, and unilateral edema in 5 per cent. Since the veins were not often dissected out, it is not known how many of these cases were due to venous thrombosis. Edema of the legs may also be caused by ascites as well as by neoplastic invasion of the vena cava or iliac veins. None of the investigations dealing with terminal phlebotrombosis indicate that it is peculiar to carcinoma of the pancreas.

Thrombophlebitis. In rare instances of unsuspected malignant neoplasm, thrombophlebitis may be the first symptom which attracts the patient's attention. It is generally agreed that in older persons with multiple thrombophlebitis one should consider the possibility of a malignant neoplasm. Woolling and Schick¹⁵ found an initial thrombophlebitis in 15 cases of unsuspected malignant disease.

Does thrombophlebitis suggest carcinoma of the pancreas? Sproul¹⁶ found multiple venous thrombi in 2 of 81 cancers of the lung and in 2 of 147 cancers of the stomach, but in 8 of 47 cancers of the pancreas. Woolling and Schick¹⁵ found the cancer in the pancreas in 3 of 15 cases. Wright¹⁷ stated that the cancer is frequently in the pancreas but may arise in various other organs. Leach¹⁸ found that venous thrombosis was not a prominent feature in his 39 cases of pancreatic carcinoma. Our records show an initial thrombophlebitis in only 9 of 609 pancreatic carcinomas (Table IV). The available data indicate that thrombophlebitis is seldom a conspicuous feature of pancreatic carcinoma.

Does thrombophlebitis associated with pancreatic carcinoma indicate that the neoplasm is situated in the body or tail? The widely quoted paper by Sproul¹⁶ is based upon the finding of thrombophlebitis in 5 of 16 cases of carcinoma of the body and tail and in only 3 of 31 cases of carcinoma of the head. Kenney¹⁹ reported two cases, one carcinoma of the body and one carcinoma of the tail, associated with thrombophlebitis. Gore²⁰ believes that the correlation of carcinoma of the body or tail with thrombophlebitis is significant. In two cases of thrombophlebitis, Oelbaum and Strich²¹ found one carcinoma in the tail and the other in an undetermined site. Wright¹⁷ stated that in his experience the site of the tumor in the pancreas was unimportant. The above data are inadequate for statistical study. The nine cases of initial thrombophlebitis listed in Table IV do not suggest that the site of the tumor in the pancreas is important. Still less significant are the reports that arterial thrombosis (Buttross and Salatch²²) and terminal endocarditis (Smith and Yates²³) are related to pancreatic carcinoma.

Ascites

Ascites is an occasional presenting symptom in carcinoma of the pancreas, and at necropsy it was found in 54 per cent of the subjects. Moderate ascites, less than 1,000 cc. of fluid, was found in 23 per cent; and severe ascites, 1,000 to 5,000 cc., was observed in 31 per cent. When the cases are arranged according to the anatomical types it is found that there are no significant differences in the incidence or severity of ascites in the different groups. Among the important causes of ascites are thrombosis or compression of the portal vein, massive metastases in the liver, and peritoneal metastases; but frequently no adequate explanation was found. Ascites was occasionally noted when there were no gross metastases.

Metastases

The incidence and distribution of gross metastases found at necropsy are shown in Table VI. It should be emphasized that these are only the grossly visible metastases. The absence of gross metastases does not mean that the patient could have been cured by pancreatectomy, since it is practically impossible to exclude the presence of microscopic metastases at necropsy. We know that these tumors usually recur after removal of all visible tumor tissue. It must also be emphasized that the necropsy discloses only the end-stage of the disease and does not tell us when the metastases occurred. However, in the carcinomas that do not produce jaundice it is highly probable that the initial symptoms are usually due to extension of the cancer beyond the pancreas.

The distribution of the metastases of carcinomas of the body and tail and the diffuse type were so similar that the three forms have been combined in Table VI for comparison with carcinoma of the head. It is noted that gross metastases were absent in 25 per cent of carcinomas of the head but in only 5 per cent of the other three types. This has been noted by others (Russum and Carp²⁴). It may mean that carcinomas of the body and tail metastasize earlier, but it probably indicates that they are present a longer time before fatal complications develop.

The more common sites of metastases in order of frequency are the liver, the regional nodes about the pancreas, the peritoneum, and the lungs. In necropsy reports, metastases are found in the liver in about two thirds of the cases. In most of the organs, metastases are less frequent from carcinoma of the head than from carcinoma of the body or tail (Table VI).

TABLE VI

Distribution and Frequency of Metastases in the Four Anatomical Types of Carcinoma of the Pancreas

	Metastases						
	Head		Body	Tail	Diffuse	Total no.	
	No. of cases	%	No. of cases	No. of cases	No. of cases	Body, tail diffuse	%
Total number	360		111	45	93	249	
No gross metastases	93	25.8	6	2	5	13	5.2
Liver	212	58.9	75	32	62	169	67.9
Regional lymph nodes	137	38.0	51	14	41	106	42.6
Peritoneum	67	18.6	45	18	37	100	40.2
Lungs	59	16.4	31	10	26	67	26.9
Adrenal glands	27	7.5	15	5	11	31	12.4
Duodenum	24	6.7	4	0	4	8	3.2
Kidneys	12	3.3	8	3	8	19	7.6
Stomach	9	2.5	16	3	7	26	10.4
Gallbladder	8	2.2	1	6	2	9	3.6
Spleen	7	1.9	4	1	6	11	4.4
Bones	6	1.7	4	0	6	10	4.0
Pleura	6	1.7	6	3	8	17	6.8
Brain and meninges	6	1.7	2	2	4	8	3.2
Skin	5	1.4	2	0	1	3	1.2
Heart and pericardium	4	1.1	2	2	4	8	3.2
Ovaries	4	1.1	2	2	0	4	1.6
Mediastinal lymph nodes	4	1.1	2	0	2	4	1.6
Peripheral lymph nodes	2	0.5	1	1	2	4	1.6
Thyroid gland	2	0.5	1	0	1	2	0.8
Uterus	2	0.5	2	0	0	2	0.8
Urinary bladder	2	0.5	0	0	0	0	0.0
Testes	1	0.3	0	0	0	0	0.0
Spinal cord	1	0.3	1	1	1	3	1.2

TABLE VII

The Incidence of Cholelithiasis in Subjects with Carcinoma of the Pancreas

	Carcinoma of head		Body		Tail		Diffuse		All types	
	Total no.	Percentage with cholelithiasis	Total no.	Percentage with cholelithiasis	Total no.	Percentage with cholelithiasis	Total no.	Percentage with cholelithiasis	Total no.	Percentage with cholelithiasis
Males	205	13.2	68	11.8	29	17.2	58	17.2	360	13.9
Females	96	39.6	29	41.4	7	42.9	21	23.8	153	37.9
Total	301	21.6	97	20.6	36	22.2	79	19.0	513	21.0

Cholelithiasis

The incidence of gallstones in the several anatomical types of pancreatic carcinoma is shown in Table VII. Gallstones were found at necropsy in about 15 per cent of males and 25 per cent of females of the older age groups. The incidence in females shown in Table VII is higher than is usually reported. When the data for males and females are combined, it becomes apparent that the incidence of gallstones is unrelated to the anatomical type of cancer. Obstruction of the common bile duct does not increase the frequency of cholelithiasis. We may conclude that carcinoma of the pancreas is unrelated to cholelithiasis.

SUMMARY OF PART I

This study comprises 609 cases of carcinoma of the pancreas necropsied at the University of Minnesota during a period of 45 years, 1911 to 1954. The percentage of deaths in males over 40 years of age due to this neoplasm increased from 0.98 per cent in the period 1911 to 1944 to 1.57 per cent in the period 1945 to 1954. In females during the corresponding periods the increase was from 1.03 to 1.19 per cent.

Carcinoma of the pancreas in males comprised 5 per cent of the fatal neoplasms during the period 1911 to 1944, and 6 per cent during the period 1945 to 1954. This increase appears to be due to the great increase in the number of old men, since carcinoma of the pancreas in males reaches a maximum in the eighth decade.

The percentage of deaths in males over 40 years of age due to malignant neoplasms was 35 per cent greater in the period 1945 to 1954; in females the increase was only 12.5 per cent. Since cancer affects a much higher proportion of younger women than younger men, the increased span of life has less effect on total cancer incidence in the female.

Of the 609 cases, cancer of the head of the pancreas comprised 59.1 per cent; of the body, 18.2 per cent; of the tail, 7.4 per cent; and the diffuse type, 15.3 per cent. About one half of the patients died within 6 months after the appearance of the first symptom and 17.5 per cent survived over 1 year. There were no significant differences in the duration of symptoms in the four groups.

Abdominal pain or distress was the most frequent initial symptom in each group. Jaundice was an initial symptom in 41.9 per cent of carcinomas of the head, and developed later in an additional 40.4 per cent. In carcinomas of the body and tail, jaundice was an initial symptom in only 2 of 154 cases, but it developed late in the course

of the disease in 26 other cases. Thrombophlebitis was an initial symptom in 9 cases. The number of cases is small but there is no indication that this complication is more frequent in carcinomas of the body and tail.

Apart from the development of jaundice, there are no consistent clinical differences in the four anatomical forms of carcinoma. Distant metastases were found at necropsy in 75 per cent of carcinomas of the head and in 95 per cent of the other three types.

II. THE RELATION OF CARCINOMA OF THE PANCREAS TO DIABETES MELLITUS

It is well known that carcinoma of the pancreas is frequently associated with glycosuria and hyperglycemia, especially in advanced stages of the disease. This is the more significant since other malignant neoplasms that produce a similar emaciation often cause hypoglycemia but never hyperglycemia. Starvation of a diabetic patient reduces sugar of the blood and urine.

Glycosuria. From collected statistics, Berk⁹ found glycosuria in 9.4 per cent of cases of pancreatic carcinoma. By combining the data reported in references 6, 7, 12, 13, and 23, we find glycosuria in 77 (19 per cent) of 405 cases. In our 443 cases of carcinoma of the pancreas in which the urine was examined, glycosuria was found in 64 (14.4 per cent).

Hyperglycemia. Berk,⁹ in collected statistics, found hyperglycemia in 19.4 per cent. The combined data from references 3, 6, 12, 23, and 25 give 69 instances of hyperglycemia among 338 cases of pancreatic carcinoma—an incidence of 20.4 per cent. Data on glucose tolerance are not reviewed since this test is not dependable in emaciated subjects.

Diabetes Mellitus. The combined data from references 6, 7, 23, 26, 27, and 28 furnish 30 cases which the authors regarded as diabetes among 390 cases of pancreatic carcinoma—an incidence of 7.7 per cent. The diabetes may precede the symptoms of carcinoma, or it may appear concurrently with the signs of cancer. Sometimes it develops after the carcinoma has been diagnosed. Several of the authors seem uncertain of the diagnosis of diabetes when the signs of its presence coincide with or follow the evidences of carcinoma; but they are more confident when the diabetes precedes the carcinoma. No one would question a diagnosis of true diabetes when it was recognized several years before the signs of carcinoma developed; but it is clear that a majority of observers have found it difficult to draw a sharp line between true diabetes and the glycosuria associated with pancreatic carcinoma.

If we define diabetes mellitus as a clinical syndrome due to insufficiency of insulin, we may recognize at least three independent forms of the disease; viz., diabetes due to removal or destruction of the pancreas, steroid diabetes, and true idiopathic diabetes. Our immediate problem is to determine whether there is an increased incidence of true diabetes in subjects with pancreatic carcinoma or, conversely, whether subjects with true diabetes show an increased incidence of carcinoma of the pancreas. These two methods of approach to the problem meet with the same difficulty, viz., the distinction between true diabetes and the glycosuria associated with pancreatic carcinoma.

*The Incidence of Diabetes in Subjects with
Carcinoma of the Pancreas*

It was noted above that the combined reports of several observers give 30 cases of diabetes among 390 cases of pancreatic carcinoma—an incidence of 7.7 per cent. The several authors do not explain how they distinguished true diabetes from the glycosuria associated with carcinoma of the pancreas, and some do not insist that all of their cases were idiopathic diabetes.

In Table VIII the incidence of diabetes in the total necropsy

TABLE VIII

Incidence of Diabetes in the Total Necropsies Compared with Its Incidence in Subjects with Carcinoma of the Pancreas

Males						
Age	No. of necropsies	Diabetic cases		Carcinoma of pancreas		
		No.	%	Total No.	Diabetic cases	
					No.	%
0-20 yrs.	7,998	18	0.23	0	0	0.0
20-40 yrs.	5,492	81	1.47	13	0	0.0
Over 40 yrs.	33,367	859	2.57	412	22	5.3
Females						
0-20 yrs.	5,755	17	0.30	0	0	0.0
20-40 yrs.	4,054	86	2.12	3	0	0.0
Over 40 yrs.	16,531	905	5.47	185	16	8.6

population is compared with its incidence in subjects with carcinoma of the pancreas. It will be noted that in males over 40 years of age diabetes is twice as frequent in those with carcinoma of the pancreas as in the general necropsy population. In females the incidence of diabetes is 50 per cent greater.

The cases classified as diabetes are listed in Table IX. With the exception of the last case in Table IX, which was classified as diabetes because of the hyaline islets, they were all good clinical examples of diabetes of mild to marked severity. The patients were treated as having diabetes and usually responded to insulin. But how many of them have true idiopathic diabetes? We know that pancreatic carcinoma may cause glycosuria. When the glycosuria is mild and asymptomatic it is labeled "glycosuria"; when severe enough to require treatment it is called diabetes. If the tumor causes mild glycosuria, why may it not produce a severe glycosuria? If we exclude from the group of true diabetes the eight males and five females (Table IX) in whom the symptoms of carcinoma antedated or coincided with the discovery of the diabetes, the incidence of diabetes in males with carcinoma of the pancreas is reduced to 3.4 per cent, and in females to 5.9 per cent; and the differences are no longer significant.

If it be admitted that carcinoma of the pancreas may produce the clinical syndrome of diabetes mellitus, it then appears possible that it may cause glycosuria before other signs of its presence appear. Therefore, some of the cases in which the diabetes antedates the carcinoma by several months may not be true diabetes. It is evident that we cannot distinguish true diabetes from the glycosuria induced by pancreatic carcinoma when the diabetes is of short duration.

In what respects do the 38 cases listed in Table IX differ from diabetic patients who did not have pancreatic carcinoma? Marble²⁹ was impressed by the short duration of the diabetes; the average duration of the diabetes in his 31 cases associated with carcinoma of the pancreas was only 3.4 years. The average duration of the diabetes in our 38 cases (Table IX) was likewise only 3.4 years; and the duration was less than 1 year in ten cases. This might mean that the carcinoma shortened the lives of the diabetic patients, but it could indicate that it caused the diabetes.

How does a pancreatic carcinoma cause glycosuria? It is generally assumed that this phenomenon is due to extensive destruction of pancreatic tissue, as in acute hemorrhagic pancreatitis. The pancreatic tissue may be replaced by the neoplasm or destroyed by an associated suppurative inflammation. But this interpretation is not supported by anatomical study of the pancreas. In 85 per cent of our cases, glycosuria did not develop although the destruction of the gland was just as great in this group as in those with glycosuria. The bulk of the islet tissue is contained in the body and tail of the pancreas, and one would expect a higher incidence of glycosuria when these seg-

TABLE IX
Carcinoma of the Pancreas Associated with Diabetes

Necropsy no.	Age	Sex	Duration of diabetes	Severity of diabetes*	Duration of carcinoma	Site of carcinoma	Hyaline islets*	Renal arterio-sclerosis*	Comment
52-1312	67	M	16 yrs.	3	2 mos.	Head	-	-	Retinitis
53-2325	64	M	14 yrs.	2	18 mos.	Head	-	0	
48-997	69	F	12 yrs.	$\begin{Bmatrix} 1 \\ 3 \end{Bmatrix}$	15 mos.	Head	-	-	
47-2476	74	M	9 yrs.	$\begin{Bmatrix} 1 \\ 2 \end{Bmatrix}$	3 mos.	Head	-	0	Coronary sclerosis
54-1208	80	M	9 yrs.	$\begin{Bmatrix} 1 \\ 3 \end{Bmatrix}$	10 mos.	Head	-	0	Coronary thrombosis
25-480	45	M	7 yrs.	2	1 mo.	Head	0	-	Gangrene of toes
51-2034	73	M	7 yrs.	1	4 mos.	Head	-	0	Insulin terminally
50-1712	72	M	6 yrs.	2	8 mos.	Head	1	1	
47-801	53	F	5 yrs.	2	5 yrs.	Body	0	1	Steatorrhea for 5 yrs.
53-1574	73	M	5 yrs.	2	6 mos.	Tail	0	1	
50-291	75	F	4-5 yrs.	$\begin{Bmatrix} 1 \\ 2 \end{Bmatrix}$	2 mos.	Diffuse	-	3	Gangrene, intercapillary glomerulosclerosis
42-122	75	F	4 yrs.	1	5 mos.	Tail	0	1	
46-2193	49	M	4 yrs.	1	19 mos.	Head	0	1	
30-1795	44	M	3 yrs.	1	9 mos.	Head	-	0	
34-337	69	F	3 yrs.	2	2 mos.	Body	-	1	
38-2467	60	M	2 yrs.	2	9 mos.	Tail	0	0	

ments are involved; but this is not true. In the 64 cases (Tables IX and X) of glycosuria and diabetes the tumor involved the head in 37, the body or body and tail in 11, the tail in 6, and the entire pancreas in 10.

Carcinomas that involve the head only produce atrophy of most of the acinar tissue of the pancreas; but the islets persist in the atrophic areas and the beta cells are filled with insulin granules (Fig. 1). Islets persist unless they are actually replaced by the neoplasm, and it is not unusual to find persistent islets filled with insulin and completely surrounded by cancer cells (Fig. 2). Stobbe³⁰ called attention to this feature. In almost every case in which intact pancreatic tissue was available, well granulated islets were found. Usually, however, the islets are embedded in atrophic pancreas or neoplastic tissue and it is possible that the insulin they form does not gain access to the circulation. The mechanism responsible for the glycosuria is not understood.

Hyaline Islets

In 208 cases of carcinoma of the pancreas without glycosuria in which intact pancreatic tissue was available for study, hyaline islets were found in eight. There was no clinical evidence of diabetes in these eight cases and the structure of the renal arterioles did not support a diagnosis of diabetes. Hyaline islets were found in only five of 22 cases listed as diabetic in Table IX. There was no intact pancreatic tissue available in 16 cases. One expects to find hyaline islets in 40 per cent of elderly diabetic patients, but the group is too small to interpret.

Renal arteriolosclerosis was found in only 10 of 28 cases (Table IX) in which tissue was available. This lesion is found in over two thirds of patients with genuine diabetes in this age group (Bell³¹). In only two cases was it greater than grade 1 in degree. There was only one case of intercapillary glomerulosclerosis, whereas in patients with genuine diabetes of this age group it is found in about 20 per cent of males and 30 per cent of females. The gangrene found in three cases and the coronary sclerosis in two others suggest true diabetes. The clinical and laboratory data therefore suggest that at least one third of the cases listed in Table IX are not idiopathic diabetes but a glycosuria induced in some way by carcinoma of the pancreas.

The 26 cases regarded as simple glycosuria are listed in Table X in the order of the known duration of the glycosuria. With respect to the duration of the glycosuria they merge gradually with those listed as diabetes and no sharp separation can be made. The glycosuria

Carcinoma of the Pancreas Associated with Glycosuria

Necropsy no.	Age	Sex	Urine sugar*	Blood sugar (mg. per 100 ml.)	Duration of glycosuria	Duration of carcinoma	Site of carcinoma	Hyaline islets*	Renal arterio-sclerosis*
48-661	54	F	3	—	5 mos.	7 mos.	Head	0	0
41-511	52	M	2	186	4-5 mos.	5.5 mos.	Head	0	0
54-3145	62	M	3	High	3 mos.	Indefinite	Diffuse	—	0
44-2038	62	F	1	246	3 mos.	8 mos.	Body	—	0
51-1768	66	M	2	110	2 mos.	2 mos.	Tail	0	1
48-501	71	F	1	288	5 wks.	5 mos.	Diffuse	—	0
53-3656	78	M	2	—	1 mo.	5 wks.	Diffuse	—	—
50-1026	55	M	1	—	1 mo.	4 mos.	Head	—	1
53-972	80	F	4	—	1 mo.	5 mos.	Head	—	0
51-2580	74	M	1	—	3 wks.	6 wks.	Head	0	0
50-733	78	M	2	—	3 wks.	2 mos.	Head	—	0
36-28	74	M	3	—	2 wks.	9 mos.	Head	0	1
47-930	47	M	2	—	2 wks.	3 mos.	Head	0	0
53-2729	68	M	1	—	2 wks.	6 wks.	Body	—	—
50-1767	78	M	4	375	9 days	14 mos.	Tail	0	1
42-1043	80	M	1	—	8 days	3 mos.	Head	—	0
43-1542	61	F	4	—	5 days	6 wks.	Head	—	—
53-1190	72	F	4	368	5 days	1 mo.	Body	—	1
53-3438	79	M	2	129	3 days	1 mo.	Body	—	—
54-1671	58	M	1	—	2 days	6 mos.	Tail	0	0
52-230	73	M	2	—	2 days	1 mo.	Diffuse	0	0
40-778	40	F	—	404	1 day	9 mos.	Head	0	—
44-2151	62	M	4	400	1 day	3 mos.	Body	—	—
41-2349	71	F	1-	232	1 day	10 mos.	Tail	—	—
45-1749	79	F	4	—	? days	10 mos.	Head	—	—
54-2842	52	M	2	70	? days	9 mos.	Body	—	—
							Tail	0	0

* The numerals refer to the intensity of the process, 1 being minimal and 4 severe; — indicates no observation.

was usually of short duration but was sometimes severe. It was generally first noted late in the disease. Hyperglycemia and glycosuria were the only evidence of diabetes. There was no supporting anatomical evidence of diabetes.

These data indicate that the apparent increased incidence of diabetes in subjects with carcinoma of the pancreas does not represent an increase of true idiopathic diabetes.

The Incidence of Cancer in Diabetic Patients

There are several statements in the literature to the effect that cancer occurs more frequently in diabetic than in non-diabetic patients. Ellinger and Landsman³² reported 39 cancers among 1,280 diabetic patients, an incidence of 3.04 per cent. They stated that only four of the patients were still living but did not give the ages at death. By the use of vital statistics in New York, they concluded that there definitely is a higher incidence of cancer in diabetic subjects. Jacobson³³ stated that diabetic patients have at least one third more cancer deaths than those without diabetes. Marble²⁹ observed 256 cancers among 10,000 diabetic patients of all ages—an incidence of 2.56 per cent, but two thirds of the patients were still alive. He thought that his data indicated that cancer is more common among patients with diabetes, but was not sure of this conclusion.

The data from our necropsies are shown in Tables XI and XII. It appears that the total incidence of cancer in males over 40 years of age is about twice as large in non-diabetic as in diabetic cases, and in females there is an even greater preponderance in the non-diabetic cases. This is to be expected since every disease which shortens life shows a decreased incidence of malignant disease. The total incidence of cancer is likewise greatly reduced in tuberculosis, heart disease, and cirrhosis of the liver.

Does cancer of the pancreas constitute a larger proportion of the cancers in diabetic than in non-diabetic cases? Ellinger and Landsman³² found one carcinoma of the pancreas among 39 cancers in patients with diabetes; but Marble²⁹ found that 33 of 256 cancers in diabetic patients (13 per cent) originated in the pancreas.

The data derived from our necropsies are shown in Tables XI and XII. In Table XI it appears that all forms of cancer are greatly decreased in diabetic subjects except carcinoma of the pancreas and carcinoma of the large intestine. There is only a moderate decrease in the incidence of carcinoma of the large intestine. Carcinoma of the pancreas is more than twice as frequent in diabetic subjects. When we compare the percentage of total cancer arising in the several

A Comparison of the Total Incidence of Cancer and of the Major Forms of Cancer in Non-Diabetic and Diabetic Males Over 40 Years of Age

No. of necropsies	Non-diabetic males over 40 years of age				Diabetic males over 40 years of age			
	32,508		A 859		B 851			
	No. of cases	Percentage of necropsies	Percentage of cancers	No. of cases	Percentage of necropsies	Percentage of cancers	No. of cases	Percentage of cancers
Stomach	1,193	3.67	15.48	9	1.05	8.49	9	9.18
Colon and rectum	977	3.01	12.68	18	2.10	17.00	18	18.36
Lung	813	2.50	10.55	8	0.93	7.55	8	8.16
Prostate	711	2.19	9.23	12	1.40	11.32	12	12.24
Pancreas	390	1.20	5.06	22	2.56	20.75	14	14.28
All other cancers	3,619	11.13	46.98	37	4.31	34.90	37	37.75
Total cancer	7,793	23.70		106	12.34		98	11.52

Group A includes all cases listed as diabetic in Table IX. Group B omits 8 cases in which the symptoms of carcinoma antedated or were concurrent with the discovery of the diabetes.

TABLE XII

A Comparison of the Total Incidence of Cancer and of the Major Forms of Cancer in Non-Diabetic and Diabetic Females Over 40 Years of Age

No. of necropsies	Non-diabetic females over 40 years of age				Diabetic females over 40 years of age			
	15,656		A 905		B 900			
	No. of cases	Percentage of necropsies	Percentage of cancers	No. of cases	Percentage of necropsies	Percentage of cancers	No. of cases	Percentage of cancers
Stomach	404	2.59	9.8	8	0.88	8.8	8	9.3
Colon and rectum	568	3.64	13.8	14	1.55	15.4	14	16.3
Breast	630	4.03	15.2	10	1.10	11.0	10	11.7
Uterus	438	2.80	10.6	6	0.66	6.6	6	7.0
Pancreas	167	1.07	4.0	16	1.77	17.6	11	12.8
All other cancers	1,930	12.35	46.7	37	4.09	40.7	37	43.0
Total cancer	4,137	26.48		91	10.05		86	9.56

Group A includes all cases listed as diabetic in Table IX. Group B omits 5 cases in which the symptoms of carcinoma antedated or were concurrent with the discovery of the diabetes.

organs in non-diabetic and diabetic patients it appears that there is a slight increase in cancer of the prostate and colon, but a striking increase in the proportion arising from the pancreas in the diabetic patient.

This observation suggests that some of the diabetic subjects with carcinoma of the pancreas did not have true idiopathic diabetes but a form of diabetes produced by pancreatic carcinoma. Accordingly, group B was constructed excluding the eight cases in males in which the symptoms of carcinoma antedated or were concurrent with the discovery of the diabetes (group B, Table XI). This decreases the ratio of pancreatic to total carcinoma but the proportion is still three times as great as in non-diabetic patients, viz., 14 per cent. It was noted above that Marble²⁹ found that 13 per cent of the cancers in diabetic patients arose in the pancreas.

A similar situation obtains in females, Table XII. After exclusion of five doubtful cases, cancer of the pancreas comprises three times the proportion of total cancer in diabetic as in non-diabetic cases.

It is to be noted that carcinoma of the large intestine is the most frequent form of cancer in diabetic patients.

The prominence of cancer of the pancreas in diabetic patients is difficult to explain. Three explanations may be considered. (a) Cancer of the pancreas may produce diabetes before it manifests any other signs of its presence. (b) Diabetic patients are more prone to malignant disease of the pancreas. (c) The difference brought out above is not real but is a statistical error due to a relatively small sample. It is to be noted that carcinoma of the colon exhibits a relative increase in diabetic cases. Much more data must be collected before any firm conclusions can be established.

SUMMARY OF PART II

There is a high incidence of glycosuria and hyperglycemia in association with carcinoma of the pancreas. No sharp distinction can be made in carcinoma of the pancreas between simple glycosuria and diabetes.

If we exclude those cases of diabetes in which the symptoms of cancer antedated or were concurrent with the recognition of diabetes, then the incidence of diabetes in subjects with carcinoma of the pancreas is not significantly greater than its incidence in the general necropsy population of corresponding age.

Diabetic patients with carcinoma of the pancreas differ from other patients with diabetes in the shorter duration of the diabetes as well

as in the decreased frequency of hyaline islets in the pancreas and vascular renal changes.

Carcinoma of the pancreas produces glycosuria probably more by interference with the escape of insulin from the pancreas than by actual destruction of islets.

The total incidence of cancer in diabetic patients is less than one half its incidence in non-diabetic patients.

According to the data presently available, which are admittedly inadequate, carcinoma of the pancreas comprises three times the proportion of total cancer in diabetic patients that it does in non-diabetic patients.

REFERENCES

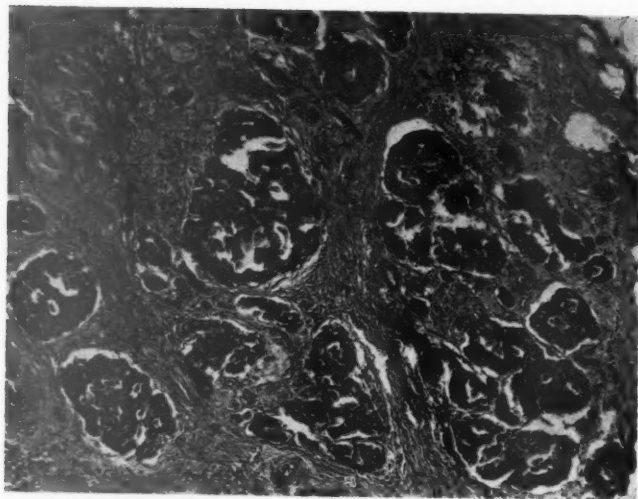
1. Corner, B. D. Primary carcinoma of the pancreas in an infant aged 7 months. *Arch. Dis. Childhood*, 1943, **18**, 106-108.
2. Grant, G. H., and Perceval, P. E. Carcinoma of the pancreas in a girl of 16, presenting as carcinomatosis of lungs. *Brit. M. J.*, 1954, **1**, 857.
3. Broadbent, T. R., and Kerman, H. D. One hundred cases of carcinoma of the pancreas: a clinical and roentgenologic analysis. *Gastroenterology*, 1951, **17**, 163-177.
4. Silver, G. B., and Lubliner, R. K. Carcinoma of the pancreas. A clinico-pathologic survey. *Surg., Gynec. & Obst.*, 1948, **86**, 703-716.
5. Duff, G. L. The clinical and pathological features of carcinoma of the body and tail of the pancreas. *Bull. Johns Hopkins Hosp.*, 1939, **65**, 69-98.
6. Country, J. C., and Foulk, R. A clinical review of thirty cases of carcinoma of the pancreas. *U. S. Armed Forces M. J.*, 1953, **4**, 831-838.
7. Brown, R. K.; Moseley, V.; Pratt, T. D., and Pratt, J. H. The early diagnosis of cancer of the pancreas based on the clinical and pathological study of one hundred autopsied cases. *Am. J. M. Sc.*, 1952, **223**, 349-363.
8. Smith, B. K., and Albright, E. C. Carcinoma of the body and tail of the pancreas: report of 37 cases studied at the State of Wisconsin General Hospital from 1925-1950. *Ann. Int. Med.*, 1952, **36**, 90-97.
9. Berk, J. E. The diagnosis of carcinoma of the pancreas. *Arch. Int. Med.*, 1941, **68**, 525-559.
10. Sanders, L. C., and McBurney, R. Carcinoma of the pancreas. *Am. J. Gastroenterol.*, 1956, **25**, 59-65.
11. Kaplan, N., and Angrist, A. The mechanism of jaundice in cancer of the pancreas. *Surg., Gynec. & Obst.*, 1943, **77**, 199-204.
12. Thompson, C. M., and Rodgers, L. R. Analysis of the autopsy records of 157 cases of carcinoma of the pancreas with particular reference to incidence of thromboembolism. *Am. J. M. Sc.*, 1952, **223**, 469-478.
13. Dashiell, G. F., and Palmer, W. L. Carcinoma of the pancreas: diagnostic criteria. *Arch. Int. Med.*, 1948, **81**, 173-183.
14. Cantor, M. O., and Haking, L. Carcinoma of the pancreas with roentgen pattern of nontropical sprue. *J. Internat. Coll. Surgeons*, 1951, **16**, 631-636.
15. Woolling, K. R., and Schick, R. M. Thrombophlebitis: a possible clue to cryptic malignant lesions. *Proc. Staff Meet., Mayo Clin.*, 1956, **31**, 227-233.

16. Sproul, E. E. Carcinoma and venous thrombosis: the frequency of association of carcinoma in the body or tail of the pancreas with multiple venous thrombosis. *Am. J. Cancer*, 1938, **34**, 566-585.
17. Wright, I. S. The pathogenesis and treatment of thrombosis. *Circulation*, 1952, **5**, 161-188.
18. Leach, W. B. Carcinoma of the pancreas. A clinical and pathologic analysis of thirty-nine autopsied cases. *Am. J. Path.*, 1950, **26**, 333-347.
19. Kenney, W. E. The association of carcinoma in the body and tail of the pancreas with multiple venous thrombi. *Surgery*, 1943, **14**, 600-609.
20. Gore, I. Thrombosis and pancreatic carcinoma. *Am. J. Path.*, 1953, **29**, 1093-1103.
21. Oelbaum, M. H., and Strich, S. J. Thrombophlebitis migrans and carcinoma of body and tail of pancreas. *Brit. M. J.*, 1953, **2**, 907-909.
22. Buttross, D., Jr., and Salatic, J. Venous and arterial thromboses in association with carcinoma of the pancreas. *Ann. Int. Med.*, 1955, **43**, 213-216.
23. Smith, J. P., and Yates, P. O. The thrombotic syndrome associated with carcinoma. *J. Path. & Bact.*, 1955, **70**, 111-117.
24. Russum, B. C., and Carp, O. Carcinoma of the body and tail of the pancreas. A clinical and pathological entity. *Am. J. Surg.*, 1942, **56**, 414-422.
25. Grauer, F. W. Pancreatic carcinoma; a review of 34 autopsies. *Arch. Int. Med.*, 1939, **63**, 884-898.
26. Arkin, A., and Weisberg, S. W. Carcinoma of the pancreas, a clinical and pathologic study of seventy-five cases. *Gastroenterology*, 1949, **13**, 118-126.
27. Levy, H., and Lichtman, S. S. Clinical characteristics of primary carcinoma of the body and tail of the pancreas. *Arch. Int. Med.*, 1940, **65**, 607-626.
28. Sloan, L. E., and Wharton, G. K. Cancer of the pancreas. *Am. J. Gastroenterol.*, 1954, **21**, 441-458.
29. Marble, A. Diabetes and cancer. *New England J. Med.*, 1934, **211**, 339-349.
30. Stobbe, H. Langerhanssche Inseln in Pankreaskarzinom. *Ztschr. ges. inn. Med.*, 1954, **9**, 917-918.
31. Bell, E. T. Renal vascular disease in diabetes mellitus. *Diabetes*, 1953, **2**, 376-389.
32. Ellinger, F., and Landsman, H. Frequency and course of cancer in diabetics. *New York State J. Med.*, 1944, **44**, 259-265.
33. Jacobson, P. H. A statistical study of cancer among diabetics. *Milbank Mem. Fund Quart.*, 1948, **26**, 90-118.

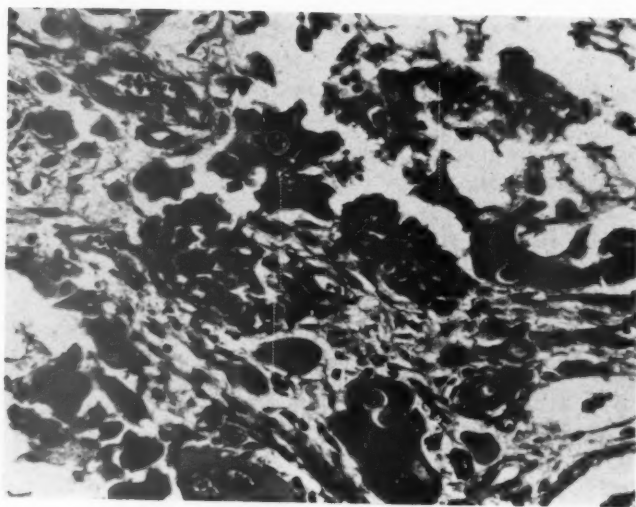
LEGENDS FOR FIGURES

FIG. 1. Area of pancreas showing atrophy of all acinar tissue, but persistence of islets. Hematoxylin and eosin stain. $\times 150$. The Gomori stain showed the beta cells filled with insulin granules.

FIG. 2. Area of pancreas showing two persistent islets in a mass of carcinomatous tissue. The beta cells are filled with insulin granules. Gomori's stain. $\times 150$.



1



2

THE ASSOCIATION OF METACHROMATIC GROUND SUBSTANCE
WITH FIBROBLASTIC ACTIVITY IN GRANULATION TISSUE*

H. E. TAYLOR, M.D., and A. M. SAUNDERS, A.B.†

*From the Department of Pathology, Faculty of Medicine,
the University of British Columbia, Vancouver 8, B.C.*

It is generally agreed that an intercellular substance, which stains metachromatically with toluidine blue, is associated with the formation of reticulin and collagen fibrils in granulation tissue.¹⁻³

The exact mechanism by which fibrils are actually formed is not clear. This would appear to be a physicochemical problem of carbohydrate-protein metabolism with which this paper is not primarily concerned, though it should be stated that at present there are three basic theories. The first supports a concept that collagen fibrils are formed extracellularly depending upon the conversion of fibrin; the second suggests that the fibrils are formed within or on the surface of the fibroblast and originate by a "splitting off" process; the final and most strongly supported theory suggests that collagen fibrils are formed extracellularly but not directly from fibrin. It is thought that certain mesenchymal cells secrete a substance or substances that can interact with components of the interstitial fluids and, possibly by a process of polymerization, result in the organization of fibrils. These various theories have been discussed recently, and at length, in a number of symposia and conferences to which one should refer for details.⁴⁻⁸

In the early stage of repair, one can demonstrate large amounts of metachromatic intercellular substance and it is assumed that this substance belongs to the group of acid mucopolysaccharides that have been distinguished by Meyer.^{9,10} It is doubtful whether the presently available histochemical methods are sufficiently accurate to allow one to identify specifically the exact nature of this substance, though it is thought to belong to the hyaluronic acid-chondroitin sulfate complexes.¹¹ There is no unanimity of opinion as to its origin. Many investigators believe that the fibroblast is concerned in its formation,¹²⁻¹⁴ and Gersh and Catchpole¹⁵ have described within fibroblasts, granules that were positive with the periodic acid-Schiff (PAS) stain and which they suggested may represent a secretion product that

* Assisted by grants from the Dr. C. J. Willoughby Research Fund and the President's Research Fund, University of British Columbia.

Presented, in part, at the Fifty-third Annual Meeting of the American Association of Pathologists and Bacteriologists, Cincinnati, Ohio, April 26, 1956.

Received for publication, July 2, 1956.

† Medical student, Research Assistant.

could become incorporated into the ground substance of connective tissue. On the other hand, Asboe-Hansen¹⁶ has accumulated considerable evidence to suggest that the metachromatic intercellular substance may be formed by connective tissue mast cells, perhaps by a process of degranulation.

In order to study the morphologic relationship between cellular activity and the formation of metachromatic material, we have utilized a modification of the method described by Lattes and Frantz.¹⁷

MATERIALS AND METHODS

Small gelfoam (absorbable gelatin sponge) pledgets were inserted subcutaneously into rats of the Wistar strain of both sexes, using sterile precautions. The pledget was placed in a trocar which was inserted through a small incision in the skin on the back of the rat and the pledget was left at a point 3 to 4 cm. distance from the opening. The incision was then closed with a small skin clip. Groups of animals were killed at 17 to 24 hours and at 2, 3, 7, and 14 days. In all, 35 rats were so treated and approximately equal numbers were in each group.

The pledget and surrounding tissue, including the overlying skin, were removed carefully and immediately fixed in either 10 per cent formalin or alcohol-formalin. Paraffin blocks were prepared and from each block, serial sections were stained as follows: hematoxylin and eosin; Hale's colloidal iron method for mucopolysaccharides as modified by Rinehart and Abul-Haj¹⁸; 0.5 per cent aqueous toluidine blue; periodic acid-Schiff (McManus' method); Wilder's silver impregnation method for reticulin, counterstained with van Gieson's stain; Masson's trichrome and Mallory's aniline blue connective tissue stains.

FINDINGS

Within 24 hours after the pledget was inserted there was a marked mesenchymal cell activity in the tissues adjacent to the pledget, including the fascial planes and overlying dermis. Usually by 18 hours large numbers of these cells had migrated to form a band about the circumference of the pledget. Many of these cells were obviously immature, plump fibroblasts, sometimes with bizarre shapes; others were smaller "round cells" that failed to exhibit phagocytosis of India ink that had been injected intravenously into three rats.

Within the next 24 hours in the sections stained for acid mucopolysaccharides, varying quantities of intercellular material were observed about the pledget, lying between the cells. This was metachromatic in the sections stained with toluidine blue and it was deep blue in those stained by the Hale colloidal iron method. In some instances

it formed a striking band completely encircling the pledget (Fig. 1), but in many cases it was patchy; some areas were strongly positive, others negative. When examined under high magnification, the material appeared as amorphous blobs in close relation to the cells (Fig. 2). Occasionally minute spherical granules were observed in it, giving the same staining reactions. This intercellular material did not react with the PAS reagent. In 24 to 48 hours it commenced to show a fibrillar appearance though it continued to react positively with the colloidal iron stain and remained metachromatic to toluidine blue (Fig. 3).

In the neighboring dermis there were mast cells, their cytoplasm staining bright blue with the Hale stain, and showing metachromatic granules with toluidine blue. Only a very occasional similar cell was noted in the cellular reaction about any of the pledgets. Because it was possible that the intensely staining intercellular substance (particularly with Hale's stain) might obscure mast cells in the background, a number of sections were treated with testicular hyaluronidase* (15 turbidity units per ml. of 0.3 per cent sodium chloride, pH 6.3; with controls in 0.3 per cent sodium chloride alone), since it has been noted that this enzyme will inhibit the metachromasia of the intercellular material but does not affect the staining of the cytoplasmic granules of the mast cells.^{12,14,19} In the sections so treated all metachromasia was inhibited in the intercellular substance and this also failed to stain with the colloidal iron; again the mast cells gave positive reactions and stood out sharply in the dermis, but only an occasional one was seen in the zone of cellular reaction.

A careful comparison was made of the serial sections stained by hematoxylin and eosin and colloidal iron, in each instance specific areas being marked on each slide. In those areas showing amorphous stainable intercellular mucopolysaccharide, one could see in the immediately adjacent slide stained by hematoxylin and eosin, immature fibroblasts which were plump and ovoid, and occasionally of bizarre shape. Intermingled with these were greater numbers of ovoid mesenchymal cells whose exact type could not be distinguished, though it is believed that they mature into fibroblasts (Fig. 6). On the other hand, in areas where either no intercellular substance was visualized or where it had assumed a fibrillar character, the majority of cells had matured as classical spindle-shaped fibroblasts and fine fibrils could be seen in their neighborhood (Fig. 7).

These features were most striking in the rats containing pledgets that had been present 48 to 72 hours, in which a double zonal effect about the periphery of the pledget often was observed. This double

* Kindly supplied by Wyeth Laboratories, Philadelphia, Pa.

zone represented an inner layer of young reacting cells immediately next to the pledget with an outer layer of fibroblasts which were obviously older and mature (Fig. 8). In such cases observation of a serial section stained with colloidal iron or toluidine blue showed a band of stainable intercellular material in the inner zone adjacent to the pledget. Outside of this zone (i.e., more distant from the periphery of the pledget) no intercellular substance was visualized, but distinct fibrils were present, presumably of collagenous nature inasmuch as they stained red with the picro-fuchsin counterstain of the Hale method (Fig. 4). Adjacent sections treated by Wilder's silver impregnation method showed reticulin fibers in the inner zone (Fig. 5), where the intercellular substance had been demonstrated. These reticulin fibers sometimes were coated with stainable mucopolysaccharides while collagen fibers made up the older outer zone. Comparison of the cellular reaction corresponding to these two zones revealed the usual picture of immature fibroblasts and admixed "round cells" next the pledget, while in the outer zone the vast majority of cells were mature classical fibroblasts with fibers (Fig. 8). Only occasional mast cells were seen in either zone.

In those rats in which the pledget had been implanted for 1 and 2 weeks, there was a good band of fibrous tissue with foreign body giant cells about the sponge; otherwise, cell activity was now minimal. Mast cells could still be seen both in the dermis and in the newly formed fibrous tissue, but no stainable intercellular substance was visible in either situation.

DISCUSSION

The morphologic findings of this study have been interpreted to indicate that the stainable acid mucopolysaccharides of the intercellular substance are formed early in the process of repair when the majority of reacting mesenchymal cells are immature and some are just assuming the morphologic appearances of young fibroblasts. As these cells quickly mature and become classical fibroblasts, the stainable mucopolysaccharides assume a fibrillar appearance and then disappear. In their place are fibers, first like reticulin and later like collagen.

We were unable to demonstrate an association between the intercellular acid mucopolysaccharides and tissue mast cells. The latter, while quite numerous in the neighboring dermis, were never conspicuous in the zone of granulation tissue. If it were argued that the metachromatic material resulted from a degranulation or "secretion" of the mast cells, then one would expect to find excessive numbers of characteristic granulated mast cells about the periphery of a pledget in an earlier phase of the process. This was never observed. Further-

more, it should again be emphasized that the cytoplasmic acid mucopolysaccharides of the mast cells, while staining in a manner similar to that of the intercellular material, react quite differently to testicular hyaluronidase.

It is our belief that the metachromatic intercellular substance is formed through the influence of immature fibroblasts and only during a relatively short period of their physiologic activity. During this phase of their development they are largely ovoid and resemble to some extent the so-called round cells of an inflammatory infiltrate, which Hartwell²⁰ emphasized in his studies of wound healing in humans. However, we do not agree with Hartwell that they are lymphocytes of hemal origin, but believe that they are immature connective tissue cells which have migrated from the pre-existing tissue planes to the site of repair and which quickly mature to classical spindle fibroblasts.

The observation that the stainable metachromatic substance disappears as fibers are formed is, of course, not new. Recently, Dunphy and Udupa²¹ have correlated chemical and histochemical sequences in wound healing in Wistar rats. They estimated the glucosamine and hydroxyproline contents of healing wounds, and assumed that the former gave an indication of the content of metachromatic ground substance and that the latter indicated collagen. They found an inverse ratio between the two. In the early phase of healing there was a high glucosamine and a low hydroxyproline content while in the later phase the converse obtained. On the basis of their findings they suggested that wound healing has two phases: a productive or substrate phase during which acid mucopolysaccharides and soluble protein precursors of collagen are produced, and a collagen phase characterized by fibril formation. This agrees entirely with the morphologic observations reported here.

The converse of this normal process is well known in scurvy and has been reported in detail by Penney and Balfour¹⁴ and by Bunting and White,¹³ who observed that wounds in partially scorbutic animals were characterized by increased metachromatic ground substance and negligible collagen formation, thus simulating the early phase of wound healing.

Lattes and his co-workers,²²⁻²⁴ in a number of papers dealing with the influence of cortisone on wound healing, reported a decreased formation of stainable metachromatic ground substance in the wounds of rats receiving 15 mg. of cortisone acetate intramuscularly for 3 days before injury and daily thereafter until killed. They suggested that cortisone in some way interferes with the production of the acid mucopolysaccharides of ground substance and consequently with sub-

sequent collagen formation. In later experiments²³ they were able to modify this inhibitory influence of cortisone by impregnating pledgets of cotton daily, after implantation, with acetyl-glucosamine and two polymers of hyaluronic acid. In such experiments they observed a moderately good reparative response in the cortisone treated animals, thus substantiating the hypothesis that these acid mucopolysaccharides are of importance in the initiation of the repair process.

Watson and Pearce²⁵ have reported their findings dealing with the cutaneous mucopolysaccharides in a patient with localized pretibial myxedema. Control specimens of skin from legs gave values of 24.5 mg. per cent of hyaluronic acid (H.A.) and 26.2 mg. per cent of chondroitin sulfate (C.S.). Compared with this, tissues taken for biopsy from the patient in question gave values of 270 mg. per cent (H.A.) and 160 mg. per cent (C.S.) while 2 years later the values were 63.6 mg. per cent (H.A.) and 48.7 mg. per cent (C.S.). The interesting feature was the fact that at the earlier date the excessive amounts of ground substance oozed from the freshly incised skin as a viscid gelatinous fluid, but at the later date the skin was hard and rubbery, and dense collagenous tissue had obliterated the zone previously filled with ground substance. The authors concluded that a progressive cutaneous fibrosis was associated with the diminishing concentration of the mucopolysaccharides.

Therefore, considerable evidence exists to support the conclusion that there is a reciprocal arrangement between the formation of intercellular metachromatic ground substance and the subsequent formation of collagen, but the exact physicochemical reactions governing the formation of fibers are not clear. Szent-Gyorgyi²⁶ once remarked that "There is no anatomy and chemistry; chemistry is anatomy at the atomic level." No truer statement has been made, and one must go to the biochemist and biophysicist to seek an explanation for the disappearance of the stainable metachromatic intercellular substance during the process of fibrogenesis.

As previously mentioned, the chemical constituents of the intercellular substance that are metachromatic to toluidine blue and give a positive staining reaction by Hale's method are acid mucopolysaccharides, i.e., polymers of glucuronic acid with glucosamine or galactosamine, and these may or may not be sulfated. According to Pearce,²⁷ the Hale colloidal iron method depends for its staining reaction on the combination of dialyzed iron with the sulfate groups of these mucopolysaccharides or with the uronic acid groups (carboxyl groups) of hyaluronic acid where this occurs in its non-sulfated form.

On the other hand, the metachromasia exhibited by toluidine blue depends upon the attraction by these same acid groups of the basic

groups on the dye molecule, thereby forming aggregates of the dye. According to Michaelis,^{28,29} these aggregates absorb light at a different range in the color spectrum and thus appear metachromatic. This hypothesis has been confirmed recently by Carnes and Forker³⁰ in their study of metachromasia of amyloid.

It should be pointed out that sulfation per se is not essential for the metachromatic reaction as proved by the work of Hayashi *et al.*,³¹ who isolated hyaluronic acid and showed it to be metachromatic with toluidine blue. However, the sulfate provides a stronger negative charge which is thought to make the metachromasia more stable. This concept is supported by Mowry,³² who succeeded in increasing the metachromasia in a normally non-metachromatic polysaccharide (dextran) by increasing its sulfate content. It is interesting to note that as the metachromasia increased in Mowry's study, the PAS response decreased. This feature of a metachromatic substance being PAS negative has been referred to by both Pearse²⁷ and McManus,¹¹ while, conversely, Hayashi and his co-workers³³ have shown that a decrease in metachromasia of mucopolysaccharides through the action of testicular hyaluronidase is accompanied by an increased reactivity to the PAS stain. The PAS reaction depends on available free 1:2 glycol groupings; if these are blocked (for example, by sulfate groups), the reaction will be negative. Kramer and Windrum³⁴ have extended this work by a histochemical technique involving the sulfation of various tissue polysaccharides, thus rendering them metachromatic. In summary it appears probable that both the metachromatic and Hale colloidal iron reactions depend upon the availability of negatively charged groups, carboxyl or sulfate, on the acid mucopolysaccharides.

It has been noted already that the acid mucopolysaccharides of the intercellular substance, demonstrated by toluidine blue and Hale's staining procedures, were only visible in the early phase of repair and disappeared quickly as the fibroblasts matured and fibers were formed. This disappearance of stainable intercellular material could have a number of chemical explanations. The material might have been resorbed or it might have been altered so that it no longer reacted with the stains; for instance, the acidic groups upon which the staining reactions depend may have become hidden internally during a process of polymerization of the mucopolysaccharides. More likely, however, the active acidic groups may have become bound by basic groups of tissue origin and would thus be unavailable for the staining reactions. Such groups might be various basic amino acids known to exist in abundance in reticulin, pro-collagen, and collagen.^{7,35,36} It seems reasonable to theorize, therefore, that the acid mucopolysaccharides formed early in the process of repair play a

physicochemical rôle in the subsequent organization of collagen fibers, and in so doing lose their characteristic staining reactions.

CONCLUSIONS AND SUMMARY

In a study of the formation of fibrous tissue about gelfoam pledgets, implanted subcutaneously into Wistar rats, it was observed that an intercellular substance, metachromatic with toluidine blue and beautifully shown with Hale's colloidal iron technique, appeared early when immature fibroblasts were predominant in the cellular reaction. No evidence was found to support the theory that this substance is formed by tissue mast cells.

As the fibroblasts matured and reticulin and collagen fibers formed, the intercellular substance could no longer be demonstrated by the staining techniques used. It is suggested that the acid mucopolysaccharides comprising this metachromatic intercellular substance probably play a physicochemical rôle in the organization of fibers and in so doing lose their reactive groupings on which their staining reactions depend.

The use of color photomicrographs in this paper was made possible by a grant from the Medical Board Fund of the Vancouver General Hospital, which we gratefully acknowledge.

REFERENCES

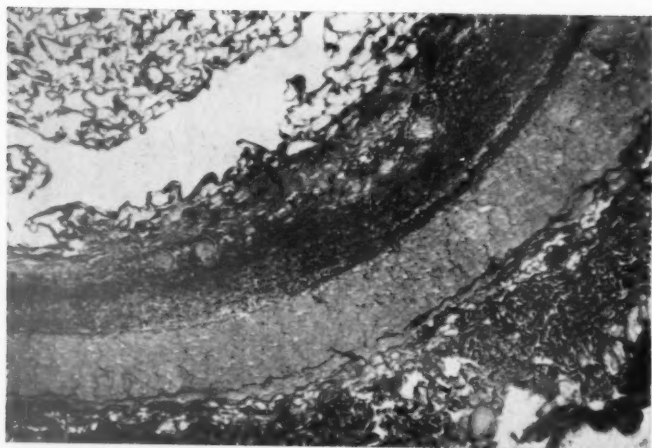
1. Ham, A. W. *Histology*. J. B. Lippincott Co., Philadelphia, 1953, ed. 2, pp. 98-108.
2. Angevine, D. M. Structure and Function of Normal Connective Tissue. In: *Connective Tissues*, Ragan, C. (ed.). Transactions of the First Conference. Josiah Macy, Jr. Foundation, New York, 1951, pp. 13-43.
3. Robb-Smith, A. H. T. The Functional Significance of Connective Tissue. In: *Lectures on General Pathology*, Florey, H. (ed.). W. B. Saunders Co., Philadelphia, 1954, pp. 553-573.
4. Gersh, I. Some Functional Considerations of Ground Substance of Connective Tissues. In: *Connective Tissues*, Ragan, C. (ed.). Transactions of the Second Conference. Josiah Macy, Jr. Foundation, New York, 1952, pp. 11-44.
5. Porter, K. R. Repair Processes in Connective Tissues. In: *Connective Tissues*, Ragan, C. (ed.). Transactions of the Second Conference. Josiah Macy, Jr. Foundation, New York, 1952, pp. 126-158.
6. Jackson, S. F. Fibrogenesis *in Vivo* and *in Vitro*. In: *Nature and Structure of Collagen*, Randall, J. T. (ed.). Butterworths Scientific Publications, London, 1953, pp. 140-151.
7. Jackson, S. F., and Randall, J. T. The Reconstitution of Collagen Fibrils from Solution. In: *Nature and Structure of Collagen*, Randall, J. T. (ed.). Butterworths Scientific Publications, London, 1953, pp. 181-191.
8. Robb-Smith, A. H. T. Normal Morphology and Morphogenesis of Connective Tissue. In: *Connective Tissue in Health and Disease*, Asboe-Hansen, G. (ed.). E. Munksgaard. Copenhagen, 1954, pp. 15-30.

9. Meyer, K. Chemistry of Connective Tissue, Polysaccharides. In: *Connective Tissues*, Ragan, C. (ed.). Transactions of the First Conference. Josiah Macy, Jr. Foundation, New York, 1951, pp. 88-100.
10. Meyer, K. The Chemistry of the Ground Substances of Connective Tissue. In: *Connective Tissue in Health and Disease*, Asboe-Hansen, G. (ed.). E. Munksgaard, Copenhagen, 1954, pp. 54-69.
11. McManus, J. F. A. Histochemistry of Connective Tissue. In: *Connective Tissue in Health and Disease*, Asboe-Hansen, G. (ed.). E. Munksgaard, Copenhagen, 1954, pp. 31-53.
12. Bunting, H. The distribution of acid mucopolysaccharides in mammalian tissues as revealed by histochemical methods. *Ann. N.Y. Acad. Sc.*, 1950, **52**, 977-982.
13. Bunting, H., and White, R. F. Histochemical studies of skin wounds in normal and in scorbutic guinea pigs. *Arch. Path.*, 1950, **49**, 590-600.
14. Penney, J. R., and Balfour, B. M. The effect of vitamin C on mucopolysaccharide production in wound healing. *J. Path. & Bact.*, 1949, **61**, 171-178.
15. Gersh, L., and Catchpole, H. R. The organization of ground substance and basement membrane and its significance in tissue injury, disease and growth. *Am. J. Anat.*, 1949, **85**, 457-521.
16. Asboe-Hansen, G. Hormonal Effects on Connective Tissues. In: *Connective Tissues*, Ragan, C. (ed.). Transactions of the Fifth Conference. Josiah Macy, Jr. Foundation, New York, 1954, pp. 123-182.
17. Lattes, R., and Frantz, V. K. Absorbable sponge tests. *Ann. Surg.*, 1945, **121**, 894-896.
18. Rinehart, J. F., and Abul-Haj, S. K. An improved method for histologic demonstration of acid mucopolysaccharides in tissues. *A. M. A. Arch. Path.*, 1951, **52**, 189-194.
19. Taylor, H. E. The role of mucopolysaccharides in the pathogenesis of intimal fibrosis and atherosclerosis of the human aorta. *Am. J. Path.*, 1953, **29**, 871-883.
20. Hartwell, S. W. The Mechanisms of Healing in Human Wounds. Charles C Thomas, Springfield, Ill., 1955, pp. 59-71.
21. Dunphy, J. E., and Udupa, K. N. Chemical and histochemical sequences in the normal healing of wounds. *New England J. Med.*, 1955, **253**, 847-851.
22. Lattes, R.; Blunt, J. W., Jr.; Rose, H. M.; Jessar, R. A.; Vaillancourt, de G., and Ragan, C. Lack of cortisone effect in the early stages of inflammation and repair. *Am. J. Path.*, 1953, **29**, 1-19.
23. Lattes, R.; Jessar, R. A.; Meyer, K., and Ragan, C. Factors modifying the cortisone-induced depression of inflammation and repair. (Abstract.) *Am. J. Path.*, 1953, **29**, 598-599.
24. Lattes, R.; Martin, J. R., and Ragan, C. Suppression of cortisone effect on repair in the presence of local bacterial infection. *Am. J. Path.*, 1954, **30**, 901-911.
25. Watson, E. M., and Pearce, R. H. The cutaneous mucopolysaccharides in localized (pretibial) myxedema. *Ann. N.Y. Acad. Sc.*, 1950, **52**, 1004-1005.
26. Szent-Gyorgyi, A. In: *Connective Tissues*, Ragan, C. (ed.). Transactions of the First Conference. Josiah Macy, Jr. Foundation, New York, 1951, p. 36.
27. Pearse, A. G. E. Histochemistry. J. & A. Churchill Ltd., London, 1953, ed. 1, pp. 150-151.

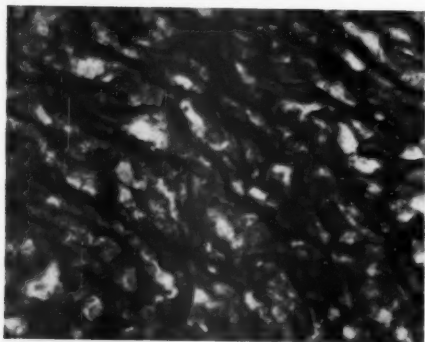
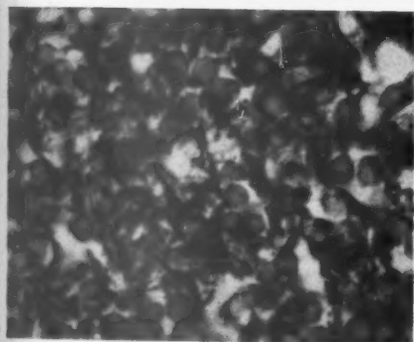
28. Michaelis, L. The nature of the interaction of nucleic acids and nuclei with basic dyes. *Cold Spring Harbor Symp. Quant. Biol.*, 1947, 12, 131-142.
29. Michaelis, L. Reversible polymerization and molecular aggregation. *J. Phys. & Colloid Chem.*, 1950, 54, 1-17.
30. Carnes, W. H., and Forker, B. R. Metachromasy of amyloid. A spectrophotometric study with particular reference to the dye-chromatrophe bond. *Lab. Investigation*, 1956, 5, 21-43.
31. Hayashi, H.; Funaki, T.; Udaka, K., and Kato, Y. Studies on acid mucopolysaccharides of the ground substance of connective tissue (report I). *Mie M. J.*, 1955, 4, Suppl. 2, 143-158.
32. Mowry, R. W. Cited by McManus,¹¹ p. 36.
33. Hayashi, H.; Udaka, K.; Funaki, T., and Inoue, T. Studies on acid mucopolysaccharides of the ground substance of connective tissue (report II). *Mie M. J.*, 1955, 4, Suppl. 2, 159-167.
34. Kramer, H., and Windrum, G. M. Sulphation techniques in histochemistry with special reference to metachromasia. *J. Histochem. & Cytochem.*, 1954, 2, 196-208.
35. Brown, G. L.; Kelly, F. C., and Watson, M. Quantitative Paper Chromatography of Amino Acids in Collagen. In: *Nature and Structure of Collagen*, Randall, J. T. (ed.). Butterworths Scientific Publications, London, 1953, pp. 117-121.
36. Randall, J. T. Discussion on the Structure of Collagen. Physical and Chemical Problems of Fibre Formation and Structure. In: *Nature and Structure of Collagen*, Randall, J. T. (ed.). Butterworths Scientific Publications, London, 1953, pp. 232-239.

LEGENDS FOR FIGURES

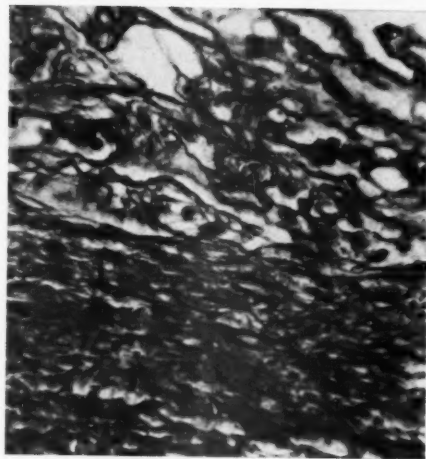
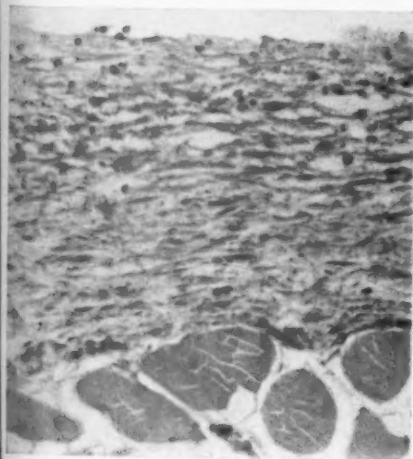
- FIG. 1. Ektachrome photograph showing an intense zone of acid mucopolysaccharides about the periphery of a gelfoam pledget seen in the upper left corner. Outside the intercellular material (blue) can be seen a muscle layer, then dermis. Pledget was implanted 48 hours. Colloidal iron method. $\times 45$.
- FIG. 2. High-power view of an area from Figure 1 to show amorphous blobs of intercellular material adjacent to cells whose nuclei stain yellow. A few fragments of disrupted pre-existing collagen (red) can be seen in the background. The cellular reaction in this area was of immature mesenchymal type (see Fig. 6). Colloidal iron method. $\times 600$.
- FIG. 3. Another area from Figure 1 to show the intercellular material becoming fibrillary. The cellular reaction here was more mature, the majority of cells being spindled fibroblasts (see Fig. 7). Colloidal iron method. $\times 500$.
- FIG. 4. Serial section to Figure 8 to show the stainable intercellular material in the inner immature cell zone (next the pledget which is above). In the outer zone where mature fibroblasts predominate only collagen fibers can be seen. Colloidal iron method. $\times 200$.
- FIG. 5. Serial section to Figure 4 to show reticulin fibers in a zone containing stainable intercellular material with collagen in the outer, more mature zone. Wilder's silver impregnation method counterstained with van Gieson's stain. $\times 375$.



1



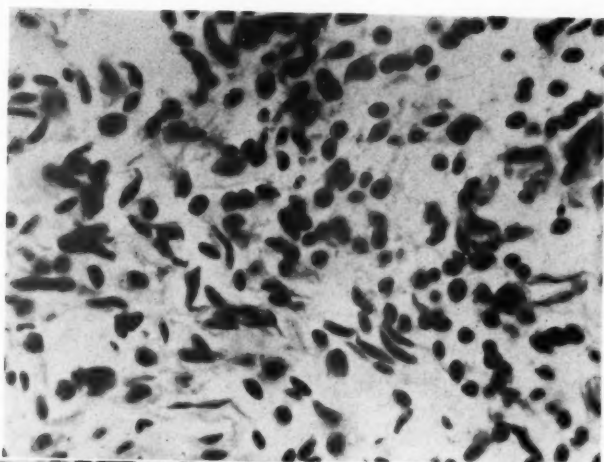
3



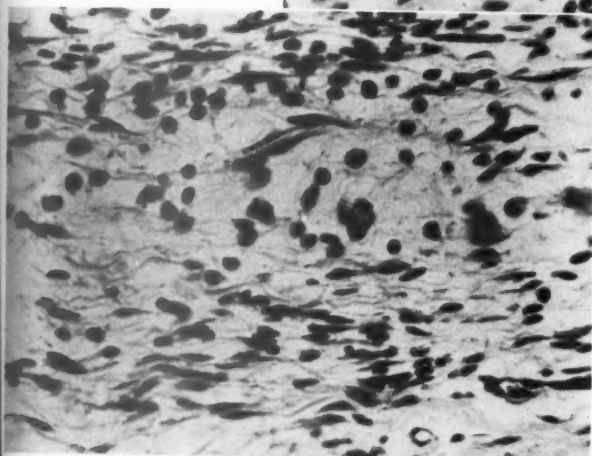
5

- FIG. 6. This photomicrograph shows the cellular reaction present in the section adjacent to that seen in Figure 2. The majority of cells are ovoid and immature; others are obviously young fibroblasts. It is believed that the former cells mature into the latter. This was the characteristic cellular pattern seen in areas where amorphous intercellular material could be demonstrated. Hematoxylin and eosin stain. $\times 425$.
- FIG. 7. This is the cellular reaction in the area seen in Figure 3. The majority of cells have now matured to classical fibroblasts and fibril formation is apparent. Hematoxylin and eosin stain. $\times 425$.
- FIG. 8. The pledget in this instance is above and outside the field photographed and was present for 72 hours. The double zone of cellular reaction is seen clearly. Near the pledget the cells are immature, while farther out they are mature fibroblasts. For comparison with Figures 4 and 5. Hematoxylin and eosin stain. $\times 250$.

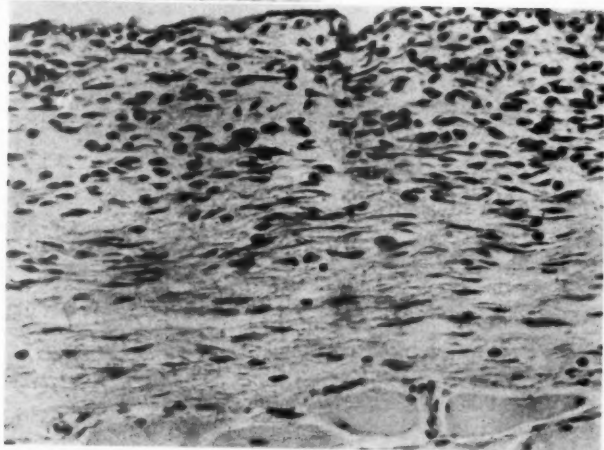
6



7



8





ANATOMICAL AND FUNCTIONAL STUDIES OF THE LUNG DEPRIVED OF PULMONARY ARTERIES AND VEINS, WITH AN APPLICATION IN THE THERAPY OF TRANSPOSITION OF THE GREAT VESSELS*

ROMEO A. VIDONE, B.S.,† and AVERILL A. LIEBOW, M.D.

*From the Department of Pathology, Yale University School of Medicine,
New Haven, Conn.*

Since it has been observed that a lung can, in time, recover a large proportion of both circulatory and respiratory function, after interruption either of its pulmonary arterial supply¹ or pulmonary venous drainage,² our interest was aroused in a study of the consequences of interrupting at a single operation both limbs of the major circulation. In the present experiments we had three objectives. (1) We wished to examine anatomically and angiographically, and to determine quantitatively, what collateral blood supply might be established. Under the conditions of the experiment this flow would represent a left to right blood shunt. (2) Our second objective was to investigate the development of retrocardiac coronary connections, and their capacity to deliver blood to the myocardium. Such arterial bridges between expanded bronchial arteries and atrial branches of the left circumflex coronary artery have been noted after ligation of the pulmonary arteries alone.³⁻⁵ The design of the present experiments would permit observation of the additional effects of limiting the outflow from the lungs. (3) Finally, we wished to observe whether, amid the complexities of the problem posed by interrupting both limbs of the major circulation, the ingrowth of vessels from both arterial and venous sides would be orderly, or confused, and thus to obtain additional insight into "what makes the vessels grow."

METHODS

Operative Procedure. Under sodium pentobarbital anesthesia (35 mg. per kg.), a midsternal approach to the contents of the thorax was employed, as described previously.⁴ The main trunk of the left pulmonary artery was tied before the homolateral veins were interrupted between ligatures. The dogs received 300,000 units of procaine penicillin G and 0.5 gm. of streptomycin on the days preceding and on the day of the operation and for 5 days postoperatively, a procedure that had been found efficient in preventing infection of the wound, and

* Supported under a contract between Yale University and the Office of Naval Research.

Presented at the Fifty-third Annual Meeting of the American Association of Pathologists and Bacteriologists, Cincinnati, Ohio, April 26, 1956.

Received for publication, July 6, 1956.

† James Hudson Brown Memorial Junior Fellow, 1954-55.

"infarction" of the lungs. The contribution of infection to this so-called infarction has been discussed in regard to the effects of ligature of the pulmonary veins.⁶⁻⁸

Determination of Pulmonary Collateral Blood Flow. The Fick principle was applied to determine blood flow in the operated lung. Bronchspirometry was performed with the aid of a rigid Van Allen cannula as in previous studies in this laboratory.^{1,2} During the bronchspirometry, the normal lung remained in free communication with the outside air, while the operated left lung was allowed to rebreathe oxygen from a spirometric chamber. Under these circumstances, it was necessary to analyze the gases in the chamber in order to take into account the nitrogen transferred into it while the oxygen was being absorbed. Bronchspirometry was continued for a 10 minute interval. Gas analyses of the blood were performed with the Van Slyke-Neill apparatus. Blood flow through the bronchial arteries (EBF) could then be calculated from the formula $EBF = a/c - b (100)$. Obviously, EBF is not the total flow through the bronchial arteries, but only that which traverses well ventilated alveoli. It is therefore a minimal value. In this formula, a = cc. of oxygen taken up by the left lung per minute, c = the oxygen concentration of blood leaving the left lung in volumes per cent, and b = the oxygen content of systemic arterial blood entering the left lung by way of the bronchial arteries. The value c cannot be determined directly, but is assumed to be the same as the systemic arterial oxygen concentration when both lungs are allowed to respire oxygen. This is actually determined after the conclusion of the bronchspirometric interval, with the cannula still in place. The tendency again is to underestimate EBF, since the actual value of c is probably less than is measured under the assumption just cited.

Determination of Collateral Blood Flow to the Heart. A dye distribution technique was used to provide an estimate of collateral blood flow to the heart. The principles and details of the actual procedure have been described elsewhere.⁵ Briefly, a measured quantity of Evans blue dye is rapidly introduced into the aorta by catheter at a point well distal to the sinuses of Valsalva, but above the orifices of the collateral vessels. The dye concentration curves in strategic parts of the circulation are determined. The early peak of dye concentration (z) in the coronary sinus that occurs before dyed blood can traverse the general circulation to reach the ostia of the coronary arteries in the proximal aorta is measured. The collateral blood flow (CBF) can then be determined as a ratio of total blood flow into the coronary sinus, by dividing z by the peak level of the dye concentration (y) in the distal aorta immediately after injection of the test dye. Then, per cent

$CBF = z/y (100)$. An alternative procedure is to compare the areas beneath appropriate segments of the respective curves (area method). Since total coronary blood flow per minute can be determined by the nitrous oxide method,⁹⁻¹² as employed by Bing and associates,^{13,14} collateral blood flow can be estimated in ml. per minute per 100 gm. of heart muscle by applying the percentage obtained from the formula to the volume of the total blood flow into the coronary sinus.

Angiography. Through the catheter within the aorta in the same position as for the dye injection study just described, between 20 and 25 cc. of 70 per cent Urokon were injected as rapidly as possible. A 50 cc. syringe compressed with the aid of a lever was capable of delivering this volume within 5 seconds. The films were exposed at intervals of 0.7 seconds.

Preparation of Casts. The procedure used in preparing the vinylite casts of the heart and pulmonary vessels has been described in detail elsewhere.^{4,15,16} In the present experiments, the azygos system and its bronchial venous tributaries also were injected.^{2,17} The sequence of the injections was as follows:

1. The thoracic aorta and its branches, including the bronchial arterial collaterals (black plastic).
2. Azygos and superior vena caval system and bronchial collateral venous tributaries (yellow plastic).
3. Coronary arteries (red plastic).
4. Right cardiac chambers and cardiac veins, including the coronary sinus (yellow plastic).
5. Left cardiac chambers and pulmonary veins (green plastic).
6. Bronchial tree (white plastic).

Steps 1 to 4 were carried out with the organs *in situ*. The entire thorax was placed in a vacuum jar and the lungs were inflated under negative pressure before completing steps 5 and 6. The cast of the bronchial tree served merely as a support for the other structures, and was made with the intent of injecting only the major bronchi, so as not to obscure detail of the vascular elements. The right lung was removed in order that the relationships of the various vessels of the heart and of the left lung might be more easily viewed.

OBSERVATIONS

Clinical Studies

The operative procedure was performed on ten dogs. The first dog died at operation from injury to a major vein. One dog was observed to produce sanguineous sputum, and died 10 hours after operation with

a massive infected infarct of the left lung. The other eight animals all survived the immediate postoperative period, five entirely uneventfully, with neither cough nor hemoptysis. Dog 370 was found dead of unknown cause at 2½ months. Dog 363 died during a study of bronchial arterial flow, probably from the effects of anesthesia.

Anatomical Studies

At necropsy, the lungs, grossly, were somewhat more shrunken and apparently more fibrous focally than after ligation of the pulmonary veins alone. Histologic sections were not made in the present series, in order to preserve the casts intact. Only in one animal was there fibrosis sufficient to prevent respiratory excursions of the lungs (dog 356).

In all instances, the pulmonary artery was found to have been interrupted without evidence of recanalization. The veins, however, had been completely interrupted in only four of the eight dogs, whereas in the others, vessels draining an estimated 10 to 60 per cent of the total volume of pulmonary parenchyma had escaped interruption (Table II). This, obviously, is a fact to consider in the interpretation of the functional data. The vein in question was usually a small branch related to a portion of one of the superior and dorsal sub-segments of the lower lobe that drains into the inferior vein.

The major arterial collateral vessels were derived from the thoracic intercostal arteries of the right side, chiefly the second to fourth (Figs. 1, 4, 5, and 6). These collaterals fed hilar plexuses, most prominent beneath the carina and about the superior surfaces of the left main bronchus. Contributing to these plexuses also were small arteries derived from the right and, more commonly, the left brachiocephalic arteries, from the internal mammary arteries, and from the pericardiophrenic branch of the latter (Figs. 5 and 11). Additional small arteries from these vessels supplied also transpleural collaterals to the lung. These must be newly formed vessels expanding from elements of granulation tissue within adhesions. In all seven of the dogs surviving more than 2 months there was retrograde injection of the still patent pulmonary arteries from the periphery by means of precapillary anastomoses, exceeding 50 μ in diameter. These vessels did not differ qualitatively or quantitatively from those observed after ligation of the pulmonary artery alone. Whatever the origin of the collateral channels, their plexiform spiral course about the bronchi was typical of that of bronchial arteries as observed in casts.¹⁸

The venous collaterals did not differ from those observed after

ligature of the pulmonary veins alone. Both expanded presumably pre-existing hilar collateral veins (Figs. 4 and 8) and newly formed transpleural collaterals were observed. The latter in particular sometimes reached a very large size (Figs. 5, 8, and 11). Venous collaterals, especially of the hilar type, but also transpleural veins derived, for example, from the pericardiophrenic vessels were seen frequently to accompany closely the greatly expanded arterial collaterals (Figs. 4, 5, and 11). This can be observed only where both the pulmonary arteries and veins have been interrupted, since arterial and venous collateral circulations are known to enlarge quite independently of one another.

The pulmonary veins on the far parenchymal side of the ligature were patent in all instances, as demonstrated by the fact of their filling with yellow plastic from the collateral veins (Figs. 5 and 8). The persisting pulmonary veins represented the actual draining vessels of the peripheral bronchi and distal air passages, and emptied at the hilum into the hilar collaterals, and at their distal ends into the transpleural collaterals.

The development of these arterial and venous collateral vessels, despite the complexity of the situation created by the ligature of both limbs of the major circulation, was orderly: bronchial arteries, even those penetrating through the pleura, always became related to the bronchi in a characteristic fashion and established connections with pulmonary arteries; and collateral veins always established connections with the still patent existing veins. No arteriovenous "short circuits" of precapillary size were demonstrated.

In a previous study of the vascular changes consequent upon cardiopneumonopexy after ligature of the pulmonary artery, large arterial connections, termed retrocardiac, were observed between the expanded bronchial collateral plexuses and the coronary arteries.⁴ These retrocardiac arteries represent enlarged continuations of the left atrial arteries, predominantly the anterior, which are branches of the left circumflex coronary artery in the dog.

Under the conditions of the present experiments, the retrocardiac vessels developed to an extent comparable to that which exists when cardiopneumonopexy has been performed also and the venous outflow of the lung has not been compromised. The anterior retrocardiac vessel usually predominated (Figs. 5, 9, and 11), but in one dog (no. 356) only a single large posterior retrocardiac artery could be demonstrated (Fig. 4). In only one animal (dog 370) were no retrocardiac arteries encountered. Details are summarized in the Appendix.

It is remarkable that, despite the usually large arterial connections, drainage of the lung into the cardiac veins was not observed.

Functional Studies

Analysis of Angiographic Data.

Of the six angiograms (Table I and Figs. 1, 2, 3, 6, 7, 9, and 10), four were made with the tip of the catheter in the desired position within the descending thoracic aorta just above the source of the major bronchial arteries. In two, the catheter had entered the ascending aorta during the manipulation of transferring the animal from the fluoroscopic to the angiographic table, but these films were, nevertheless, of interest since a coronary arteriogram (Figs. 1 and 9), as well as good opacification of the bronchial arteries, was obtained.

In the aortograms, the proximal bronchial arteries were opacified within 0.7 seconds, and the distal branches within 1.4 seconds (Figs. 6 and 10). The pulmonary artery first came into contrast between 2.8 and 4.9 seconds in all instances, and was so maintained for as long as 18.5 seconds (dog 356, Fig. 2). The pulmonary veins also were seen to receive the Urokon in three of the dogs (Fig. 3). This sometimes occurred at approximately the same time as in the pulmonary arteries, but then the veins were more faintly outlined in the initial films, indicating a slight lag in the filling of the venous side. In one animal, the interval was approxi-

TABLE I
Analysis of Angiograms

Dog no.	Interval operation to sacrifice (months)	Type of angiogram	Opacification time (seconds)					Fig. no.
			Coronary arteries	Bronchial artery, proximal	Bronchial artery, peripheral	Pulmonary artery	Pulmonary vein	Bronchial vein
362	14	Descending		0.0-0.7	1.4	4.2-4.9	4.9-5.6	
358	16	Descending		0.7	1.4	2.8		9, 10
360	16	Proximal	0.0-0.7	1.4	2.8	4.9	8.4	
361	16	Descending		0.7	1.4	4.2	4.2-4.9	6.3
371	16½	Descending		0.7	1.4	3.5	4.9	6, 7
356	17¼	Proximal	0.0-0.7	2.1	2.8	4.9	6.3	1-3

mately 1.4 seconds. Opacification of the collateral veins in continuity with the pulmonary veins was observed approximately 2.1 seconds after pulmonary venous opacification and 6.3 seconds after the dye had entered the aorta (Fig. 7). The azygos vein was prominently delineated by radio-opaque material in only one dog (no. 371) at 7.7 seconds. Lateral films are not as satisfactory as posterior-anterior films for the demonstration of this vessel, as indicated by previous experience.⁵

The sequence of events in the pulmonary circulation of the two animals that had received injections into the ascending aorta was similar to that just described, with the expected slight delay. The collateral veins ultimately became opacified at 9.9 seconds after injection in one animal (dog 356).

Evidence of opacification of the retrocardiac branches of the circumflex artery was sought in these animals. In dog 360 such a vessel was demonstrable at a time when there was only initial opacification of the bronchial arterial plexuses, with the greatest prominence at the two ends (Fig. 9). This suggests flow from the coronary to the bronchial plexuses at the moment of the radiographic exposure. The posterior retrocardiac artery was visualized in the angiograms of dog 356 at 2.1 seconds (Fig. 1). This corresponds to the one collateral connection between the coronary and bronchial arteries demonstrated in this cast.

Collateral Blood Flow to the Lungs. Blood arriving via the bronchial arteries and draining by way of collateral veins into the right heart rapidly increased in volume with time after operation. Twenty-eight observations in six dogs are recorded (Table II and Text-fig. 1). Even at 3 months, the flow had increased by a factor of at least 10, and in some instances by a factor as high as 40 times the normal as established by Bruner and Schmidt.¹⁹ At a time varying between 14 and 16.5 months postoperatively, the actual measured volumes varied from 600 to 1,410 cc. per minute. This flow may be estimated to lie in the range of one half of that to be expected were the experimental lung intact. It is apparent also that most of the increase took place within the first 3 months following operation.

Straight lines were fitted statistically* to the data from each of the dogs except no. 370, for which only two observations were available. In two cases (dogs 371 and 362) there was a statistically significant slope (bronchial flow increases with the passage of time). To obtain

* We are indebted to Dr. Colin White of the Department of Public Health for the statistical analyses.

an over-all effect, the data were pooled and two hypotheses tested. (a) Was the difference in slope from one dog to another statistically significant? In this instance $0.05 > P > 0.01$. (b) Was the over-all slope statistically significant? This is true with $P < 0.001$. Since only

TABLE II
*Effective Bronchial Collateral Blood Flow;
Left Pulmonary Arteries and Veins Ligated*

Dog no.	Weight (kg.)	Percentage of P.V. not ligated	Months postoperative	EBF arterial (ml./min.)	EBF (ml./min./kg.)	
					Arterial	Venous
370	17.5	15	1	210	11.5	9.8
			2	829	45.6	38.7
362	13.6	60	3	245	18.0	7.2
			6	345	25.3	10.1
			8	765	56.2	22.5
			14	1048	77.0	30.8
358	19.6	15	3	580	29.7	25.2
			5	614	31.5	26.8
			7.5	730	37.5	30.5
			8	675	34.6	29.0
			12	967	49.6	42.1
			16	600	30.8	26.2
360	16.8	0	2	1030	58.0	58.0
			5	621	35.3	35.3
			7.5	588	33.0	33.0
			13.5	939	52.7	52.7
			16	880	49.4	49.4
361	22.7	10	3	875	38.5	34.7
			5.25	1266	55.8	50.2
			7.25	1250	55.2	49.7
			13	1800	79.4	71.5
			16	1410	62.2	56.0
371	13.9	0	2	329	27.0	27.0
			4	584	40.3	40.3
			5	320	22.1	22.1
			7	560	38.6	38.6
			7.5	648	44.6	44.6
			16.5	840	59.7	59.7

P.V. = Pulmonary vein

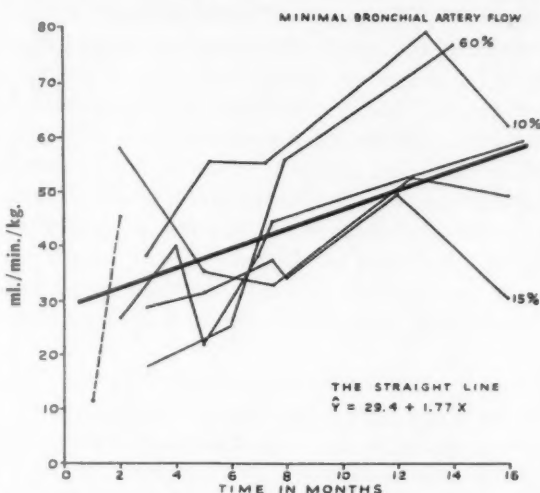
EBF = Blood flow through the bronchial arteries.

two observations were available on dog 370, the straight line in this instance was fitted statistically to the other data and may be represented as: $Y = 29.4 + 1.77X$.

In calculating bronchial venous collateral drainage as related to collateral inflow, an adjustment must be made to exclude that portion of parenchyma which had escaped interruption of venous drainage in some animals (Table II). This value exceeded 15 per cent in only a single instance (dog 362).

Collateral Blood Flow to the Myocardium. The expanded retrocardiac arteries were found by the dye distribution method to be

capable of delivering blood from the descending thoracic aorta to coronary arterioles. Such blood, thereafter, necessarily followed the course of any blood delivered to these vessels, ultimately in large measure to the coronary sinus. The volume of this collateral flow varied from 1.0 to 2.9 per cent of total inflow into the coronary sinus, as



Text-figure 1. Time, X , is plotted against Y , effective bronchial arterial flow expressed in ml./min./kg. The slope, calculated statistically, is expressed as $Y = 29.4 + 1.77X$, and is significant ($P < 0.001$). Data from dog 370, for which only two observations are available, are not included in the calculation. The percentages indicate the proportion of pulmonary substance of the left side, the draining vein of which had escaped ligation.

determined by comparison of peak dye concentrations, and between 1.6 and 3.8 per cent as determined by appropriate segments of the areas below the respective dye concentration curves (Table III). These two methods have been described elsewhere.⁵ This flow is not so large as when pulmonary arterial ligation plus cardiopneumonopexy is performed, but is significantly greater than in control animals, with no overlap of the respective values. Since the correct placement of the catheter in the coronary sinus is all important, the criteria establishing this point are indicated in Table III. The zero collateral flow recorded for the first observation in dog 360 may possibly have been an artifact resulting from a too distal position of the tip of the injection catheter in relation to the origins of the bronchial arteries. This is suspected since the collateral vessels were large in this animal (Fig. 11). Unfortunately, during the second determination, the tip of the catheter was demonstrated to be in the ascending aorta (Figs. 9 and 10) and the opposite error resulted.

TABLE III
Collateral Blood Flow to the Myocardium; Left Pulmonary Arteries and Veins Ligated

Dog no.	Interval, operation to sacrifice (months)	Date	N ₂ O flow (ml./mm. ² /100 gm.)	Collateral flow				Evidence for position of coronary sinus catheter				
				% of total		ml./min./100 gm.		Color of blood	Chest film	Sinus angiogram	Rise in sinus pressure with balloon	Necropsy
				Peak method	Area method	Peak method	Area method					
358	16	1/ 6/56 1/10/56	53	2.9 2.3	2.0 2.5	1.5 1.2	1.1 1.3	*	*	*	*	*
360	16	1/13/56 1/17/56†		0	0	0	0	*	*	*	*	*
361	16	1/21/56 1/24/56	92	1.0 1.1	2.1 1.6	0.9 1.0	1.9 1.5	*	*	*	*	*
362	16	1/27/56 1/31/56	68	2.4 2.6	3.0 3.1	1.6 1.8	2.0 2.1	*	*	*	*	*
371	16½	3/ 3/56 3/ 6/56	69	2.9 2.3	3.4 3.8	2.0 1.6	2.3 2.6	*	*	*	*	*

* Evidence present. Where there was no recorded necropsy confirmation, the catheters had been previously withdrawn.

† Injection catheter too proximal in aorta with backflow of Evans blue dye into coronary sinus. This was confirmed by the plain films and angiograms.

DISCUSSION

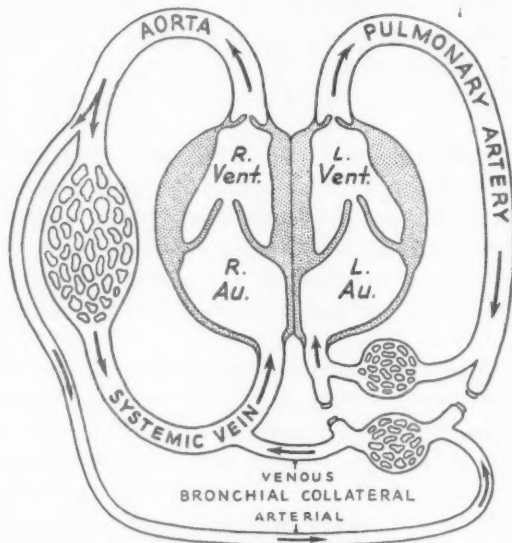
It is astonishing that ingrowth of the arterial and venous collaterals proceeds in an entirely orderly fashion to establish without fistulization connections with the corresponding pulmonary arteries and veins on the parenchymal side of the respective ligatures. This is especially remarkable in the transpleural collaterals which must be newly developed from capillaries in the granulation tissue of adhesions and cannot, therefore, be said to represent merely enlargements of pre-existing anastomoses. This orderliness cannot be explained simply on a mechanical basis since the sharpest pressure gradient is from the systemic collateral artery to the systemic collateral vein, yet the largest pre-capillary connections are always collateral artery to unobliterated pulmonary artery, and collateral vein to persistently patent pulmonary vein, and the original capillary bed of the lung is still interposed as a bridge in its original position. It is this interposition of the alveolar capillaries that permits the lungs quickly to reacquire respiratory function.

It is remarkable also that the rate of increase of collateral blood flow that is stimulated by interruption of the pulmonary artery is not strikingly diminished by the simultaneous interruption of pulmonary venous outflow. This suggests that the venous collaterals can at least keep in step with the development of the arterial collaterals—a fact previously suggested by both anatomical and functional observations.^{1,2,8,18}

The observations on the retrocardiac collaterals are of interest in that their development occurs, as might be expected, in the absence of cardiopneumonopexy. Their capacity to yield blood to the coronary arteries during at least a part of the cardiac cycle has been measured and has been found to be less than when a cardiopneumonopexy is present in addition, but without interruption of venous outflow. Comparative data regarding the effects of simple ligature of the pulmonary artery alone and of cardiopneumonopexy when both pulmonary arteries and pulmonary veins have been interrupted are still required.

An Application in the Treatment of Transposition of the Great Vessels. An important objective in the treatment of transposition of the great vessels is to bring oxygenated blood into the aorta. It has been suggested that this can be accomplished by ligature of pulmonary veins of one lung, whereby bronchial veins would carry blood from the lung to the right side of the heart.² Such a procedure would be more dangerous in transposition of the great vessels than under normal circumstances, since the pulmonary arterial pressure is elevated in approximately 50 per cent of these patients, sometimes to levels in excess

of systemic arterial pressure. When the pulmonary arteries and veins of the lung are interrupted simultaneously, however, this danger of an excessively high pulmonary capillary pressure is obviated. From comparative studies of the collateral pulmonary circulation in man under various circumstances²⁰ there is no reason to doubt that the development of the collateral circulation after the suggested operation would be as rapid as it is in the normal dog. With the development of the collaterals, there would be introduced into the systemic circuit an efficient autogenous oxygenator that receives desaturated blood from the aorta, and is competent to deliver it in a saturated state to the right atrium and, thus, to the transposed systemic circuit (Text-fig. 2). The suggested operation has the advantage that the direction of blood



Text-figure 2. The effects of ligating the pulmonary arteries and veins of one lung in transposition of the great vessels. An autogenous oxygenator, the operated lung, is introduced into the right-sided systemic circuit. This lung receives desaturated blood from the transposed aorta by way of expanded bronchial arteries, and after oxygenating it, returns it to the right heart, via expanded bronchial collateral veins. A lobe, rather than a lung, could be treated in similar fashion.

flow is inevitably in the desired direction—from left to right. On the contrary, in the usual surgical procedure, whereby a large interatrial defect or connection between the pulmonary veins and right atrium is created, blood can flow in either direction, as determined by the relative pressures on the two sides.

SUMMARY

If both the pulmonary arteries and veins of a lung are interrupted simultaneously, the ingrowth of the collaterals is entirely orderly, without fistulization among arteries and veins. The vastly expanded bronchial arteries come to supply the lung by way of precapillary anastomoses with the branches of the still patent pulmonary arteries beyond the ligature, and enlarged bronchial veins come to drain the lung by means of augmented precapillary connections with the pulmonary vein; the original alveolar capillaries continue to be interposed between the remote arterial and venous collateral limbs, a fact that accounts for the rapid recovery of respiratory function. Especially since both arterial and venous collaterals are, in part, newly formed from granulation tissue within adhesions, the existence of other than mechanical factors in guiding the collateral vessels to their proper destinations must be postulated.

The volume of collateral blood flow after ligature of the pulmonary artery is not markedly different, whether or not the pulmonary veins are simultaneously occluded, and approximates 800 ml. per minute after 16 months in a dog weighing 15 kg.

When both pulmonary arteries and veins are ligated, the collateral flow represents a large shunt of blood from the aorta to the right side of the heart. In the treatment of transposition of the great vessels, this operation, therefore, would have the effect of introducing an autogenous oxygenator into the right-sided systemic circuit. Such an operation is especially indicated since the direction of the flow of oxygenated blood is inevitably to the right side in contrast to the ambiguous result of the usual surgical procedure whereby a large interatrial septal defect is created.

In these animals, large arterial bridges also come to connect the coronary and expanded bronchial arterial systems. These are capable of contributing from 1.0 to 3.8 per cent of blood to the coronary arterial inflow as measured at the coronary sinus.

APPENDIX

DESCRIPTION OF CASTS AND ANGIOGRAMS

Dog 370

Arterial Collaterals to Lung. 1. From pericardiophrenic branch of left internal mammary. 2. From third right aortic intercostal.

There is injection only of the proximal portions of the bronchial arteries.

Collaterals to Heart. No significant retrocardiac branches observed.

Venous Collaterals to Lung. A. Hilar. 1. From common trunk of first and sec-

ond left inferior* intercostal vein a major branch accompanies bronchial artery no. 2. B. Transpleural. 2. From base of left vertebral. 3. Internal mammary. 4. Innominate.

There is retrograde injection of pulmonary veins. The pulmonary vein to the superior segment of the lower lobe has not been ligated. This accounts for approximately 15 per cent of the total mass of the left lung.

Dog 363

Arterial Collaterals to Lung. 1. From left internal mammary. 2. From second aortic intercostal. 3. From fourth aortic intercostal, this branch ascends to the level of first aortic intercostal and then descends again.

The pulmonary arteries are retrogradely injected in the lower lobe and to some extent in upper lobe.

Collaterals to Heart. A large retrocardiac vessel proceeds anteriorly from the bronchial arterial plexus. The vessels of the heart itself are not preserved.

Venous Collaterals to Lung. A. Hilar. 1. From left third inferior intercostal vein. B. Transpleural. 1 and 2. Large trunks from left vertebral vein. 3. Smaller trunk from anterior aspect of innominate vein at root of internal mammary vein.

There is complete retrograde injection of pulmonary veins.

Dog 362

Arterial Collaterals to Lung. 1. From left internal mammary artery. 2. From left brachiocephalic artery. 3. From the third right aortic intercostal artery. 4. Major trunk from fourth right aortic intercostal at the origin of latter. 5. Large trunk from the fourth right aortic intercostal artery, approximately 1.5 cm. beyond its origin.

Injection of the pulmonary arteries retrogradely is visible only in the periphery of the left lower lobe.

Collaterals to Heart. There is one large anterior retrocardiac vessel derived from the circumflex artery approximately 4 mm. beyond its origin. There is a posterior retrocardiac from the circumflex near the left ventricular margin. Another posterior retrocardiac vessel is derived from the left circumflex artery, taking origin at the source of the posterior descending branch.

Venous Collaterals to Lung. A. Hilar. No venous injection. B. Transpleural. Only a single branch from the left internal mammary artery is injected. Unfortunately, the distal left internal mammary vein was ligated during the dissection post mortem; this probably represented the major source of collateral venous drainage. The pulmonary vein of the lower lobe was not interrupted (considered 60 per cent of total substance of left lung).

Angiogram. This is a technically adequate descending aortic angiogram. 0.0 seconds: initial filling of bronchial arteries. 0.7 seconds: excellent "negative bronchogram" within the lung. 2.1 seconds: the proximal aorta is still outlined; a contribution to the bronchial arterial plexus from the phrenic artery is apparent. 4.9 seconds: the pulmonary artery is visible; bronchial arteries fading. 18.5 seconds: nothing additional is discerned; the vessel just described is still faintly visible.

Dog 358

Arterial Collaterals to Lung (Fig. 5). 1. Minute branch from brachiocephalic artery. 2. Minute transpleural branch from left internal mammary artery. 3 and 4. These represent the major sources of the arterial collateral arising close together

* This term is used for veins that accompany the aortic intercostal arteries.

from the fifth right intercostal artery. Both of these contain some red material that had entered from the coronary artery.

The pulmonary arteries are well injected retrogradely at the periphery of the lower lobe.

Venous Collaterals to Lung (Fig. 5). A. Hilar. 1. From azygos vein between the fifth and sixth inferior intercostal levels, to accompany bronchial artery no. 4. B. Transpleural. 2. From the internal mammary trunk. 3. From the left vertebral vein.

Both hilar and transpleural injection of pulmonary veins has occurred. Approximately one fourth of the lower lobe drains into a still unligated pulmonary vein (estimated at 15 per cent of the total).

Collaterals to Heart (Fig. 5). 1. A large anterior retrocardiac vessel is derived from the circumflex artery within 3 to 4 mm. of the origin of the latter. 2. A posterior retrocardiac, approximately as large as the anterior retrocardiac, originates from the circumflex artery on the posterior aspect of the heart proximal to the posterior descending, and proceeds to the left to join the bronchial arterial plexus on the medial aspect of the left lung.

Angiogram. 0.7 seconds: filling of some peripheral bronchial arterial branches has already occurred. 1.4 seconds: this filling progresses and corresponds to what is seen in the cast. 2.8 seconds: initial opacification of pulmonary artery. 4.2 seconds: bronchial arteries fading. 7.0 seconds: pulmonary arteries still visible. 15.4 seconds: no additional information.

Dog 360

Arterial Collaterals to Lung (Fig. 11). 1. From the right brachiocephalic trunk. 2. From the left vertebral artery to a plexus that lies above and behind the left main bronchus. 3. From the first left aortic intercostal artery, passing along the left side of the aorta to the posterior aspect of the left main bronchus. 4. From the second right aortic intercostal several small branches parallel to those derived from the first left aortic intercostal. 5. A major trunk from the third right aortic intercostal; this has received some red plastic from the coronary arteries. 6. From the fifth left aortic intercostal a strong branch to the subcarinal plexus; this has also received some red material near its distal end. 7. From the seventh right aortic intercostal another major branch which proceeds to the subcarinal plexus.

The lower lobe shows a very extensive arterial collateral supply, with retrograde injection of the pulmonary artery to the region of the hilum.

Venous Collaterals to Lung (Fig. 11). A. Hilar. 1. From the azygos vein at the base of the common trunk of the left third and fourth inferior intercostal veins. This vessel swings medially behind the trachea and then to the subcarinal plexus; it accompanies arterial collateral no. 5. 2. From the left fifth inferior intercostal vein a strong trunk to the subcarinal plexus. B. Transpleural. 3 and 4. Two strong branches from the left vertebral vein. There are large transpleural connections.

There is excellent retrograde injection of pulmonary veins in both upper and lower lobes; all pulmonary veins of the left side are completely interrupted at the hilum.

Collaterals to Heart (Fig. 11). 1. There is a large anterior retrocardiac branch connecting with the base of the left circumflex just beyond its first division. 2. There is a posterior retrocardiac branch from the right coronary artery which joins the left anterior retrocardiac vessel. This forms a vascular bridge that runs anteriorly of the right superior pulmonary vein, interatrial septum, and anterior wall of the left atrium.

Angiogram. In this instance, the injection of radio-opaque dye was into the

ascending arch of the aorta. 0.7 seconds: ascending aorta and arch are already filled. The right and left coronary arteries are clearly outlined. The intercostals are opacified. There is initial filling of the bronchial plexuses behind the heart and anteriorly of the aorta. 1.4 seconds: the circumflex is now visible and is in apparent continuity with the bronchial arterial plexus (Fig. 9). The coronary arteries are now fading somewhat, but the bronchial plexuses are clearly outlined. 2.8 seconds: excellent opacification of the bronchial plexuses. The source of bronchial artery no. 5 is well defined; bronchial no. 1 is visible also. The coronary arteries have faded (Fig. 10). 4.2 seconds: initial opacification of the coronary veins. 4.9 seconds: appearance of a large vessel considered to be a pulmonary artery. 6.3 seconds: bronchial arterial plexuses are fading; coronary veins and sinus are well seen, as determined by comparison with the sinus angiogram. The pulmonary artery, also, is well seen. 7.7 seconds: no change. 8.4 seconds: the pulmonary artery is still visible. The bronchial plexuses have faded. Pulmonary veins have now appeared. 12.6 seconds: lower lobe and upper lobe veins are now more clearly seen.

Dog 361

Arterial Collaterals to Lung. 1 and 2. Minute branches from the right brachiocephalic trunk. 3. A branch from the first aortic intercostal, 1.5 cm. beyond its origin. 4. A branch arising at the very root of the second right aortic intercostal.

Pulmonary arteries are injected at the periphery, especially well in the lower lobe.

Collaterals to Heart. 1. There is a small anterior retrocardiac branch arising just beyond the origin of the left circumflex. 2. The posterior retrocardiac is a large branch arising near the left margin of the heart on the posterior aspect, from the circumflex branch of the left coronary artery.

Venous Collaterals to Lung (Fig. 8). A. Hilar. 1. There is a branch that drains into the common stem of two veins at levels above vein no. 2 and which accompanies bronchial artery no. 3. 2. A branch draining into the base of the common venous ramus for the first, second, and third posterior intercostal veins, which accompanies bronchial artery no. 4. B. Transpleural. 3. From the vertebral vein. 4. From the internal mammary trunk.

The ligated pulmonary veins are injected retrogradely both from the hilar and transpleural intercostals. Approximately 10 per cent of the pulmonary substance is still drained by an unligated pulmonary vein.

Angiograms. 0.7 seconds: two large bronchial arterial trunks are visible; the dye is ascending into the proximal aorta. 1.4 to 2.1 seconds: the peripheral plexus within the lung is now better outlined. A negative bronchogram is outlined (Fig. 6). 4.2 seconds: bronchial arteries are fading; pulmonary arteries and, more faintly, pulmonary veins are becoming visible. 6.3 seconds: clearest definition of both the pulmonary arteries and veins. The ends of pulmonary veins anteriorly become tortuous, suggesting a connection with collateral veins. 8.4 seconds: further filling of collateral venous drainage from the upper lobe (Fig. 7). 16.1 seconds: these vessels are still faintly visible.

Dog 371

Arterial Collaterals to Lung. 1. From fourth right aortic intercostal artery. 2. Fifth right aortic intercostal artery.

Bronchial arterioles injected peripherally in lower lobe. No retrograde injection of pulmonary artery.

Collaterals to Heart. An anterior retrocardiac takes origin approximately 2 mm. beyond the source of the circumflex.

Venous Collaterals to Lung. A. Hilar. 1. Common stem of third and fourth left inferior intercostal veins, 4 mm. from origin, to accompany bronchial artery no. 1. 2. From azygos vein at point midway between fifth and sixth inferior intercostal levels to accompany bronchial artery no. 2. B. Transpleural. 3. From internal mammary, 4. From left side of innominate vein.

All pulmonary veins had been successfully interrupted at the hilum.

Angiogram. 0.0 to 0.7 seconds: initial filling of bronchial artery. 1.4 seconds: peripheral filling of bronchial artery. 3.5 seconds: pulmonary artery now visible. 4.9 seconds: bronchial arteries now fading. Pulmonary vein now faintly seen. Azygos vein now very faintly outlined. 7.7 seconds: azygos clearly seen for first time. 8.4 seconds: pulmonary vein more prominent than formerly. 15.7 seconds: pulmonary arteries can still be seen.

Dog 356

Arterial Collaterals to Lung (Fig. 4). 1 and 2. Two fine branches, from the left and right brachiocephalic trunks, respectively. 3. From the third right intercostal approximately 1 cm. from its source, a bronchial artery loops upwards to a level just above the second aortic intercostal to enter the subcarinal plexus. This contains some red material from the coronary arteries. 4. From the base of the third right aortic intercostal artery to the subcarinal plexuses.

In this specimen, there is only the most superficial retrograde injection of the pulmonary arteries.

Collaterals to Heart (Fig. 4). 1. Only a single posterior retrocardiac branch is identified. This is a branch of the circumflex that ascends beneath the stump of the ligated left inferior pulmonary vein to contribute to bronchial arterial plexuses anteriorly of the hilum. No anterior retrocardiac branches are present.

There is an arterial bridge from the right coronary that loops over the right atrium anteriorly of the superior vena cava and then into the interatrial septum to join a central branch of the left anterior atrial artery.

Venous Collaterals to Lung (Fig. 4). A. Hilar. 1. From the base of the common stem of the first and second inferior intercostal veins to accompany bronchial artery no. 3. 2. From the azygos vein on the left side at a point midway between the levels of the third and fourth inferior intercostal vessels to accompany artery no. 4. B. Transpleural. 1. From the left vertebral. 2. From the left internal mammary. 3. From diaphragmatic vessels.

There is an excellent retrograde pulmonary injection. All pulmonary veins had been completely interrupted at the hilum.

Angiogram. This is an ascending aortic angiogram. 0.0 seconds: initial filling of proximal ends of coronary arteries. 0.7 seconds: coronary arteries well filled; dye present in left ventricle. 1.4 seconds: no change. 2.1 seconds: the descending thoracic aorta is now well filled. Bronchial plexuses are clearly outlined. The posterior retrocardiac from the left circumflex is visibly filled. This corresponds perfectly to the cast as a posterior retrocardiac (Fig. 1). 3.5 seconds: the circumflex continues in an opacified state, as does the retrocardiac, while the other vessels are fading. 4.9 seconds: pulmonary artery initially outlined. Bronchial arteries are now fading. 5.6 seconds: pulmonary arteries now well filled (Fig. 2). 6.3 seconds: initial opacification of pulmonary veins, anteriorly of the pulmonary artery in the lower lobe. 7.7 seconds: both pulmonary arteries fading; pulmonary vein well outlined. 10.6 seconds: diaphragmatic venous collateral continuous with a pulmonary vein (Fig. 3). 14.1 seconds (last film): the venous collateral just mentioned persists but is now better shown while the pulmonary vein itself is fading.

REFERENCES

1. Bloomer, W. E.; Harrison, W.; Lindskog, G. E., and Liebow, A. A. Respiratory function and blood flow in the bronchial artery after ligation of the pulmonary artery. *Am. J. Physiol.*, 1949, **157**, 317-328.
2. Hurwitz, A.; Calabresi, M.; Cooke, R. W., and Liebow, A. A. An experimental study of the venous collateral circulation of the lung. II. Functional observations. *J. Thoracic Surg.*, 1954, **28**, 241-246.
3. Bloomer, W. E.; Stern, H., and Liebow, A. A. Application of induced pulmonary arterial collateral circulation as collateral supply to the heart. *Proc. Soc. Exper. Biol. & Med.*, 1954, **86**, 202-203.
4. Kline, J. L.; Stern, H.; Bloomer, W. E., and Liebow, A. A. The application of an induced bronchial collateral circulation to the coronary arteries by cardiopneumonopexy. I. Anatomical observations. *Am. J. Path.*, 1956, **32**, 663-693.
5. Vidone, R. A.; Kline, J. L.; Pitel, M., and Liebow, A. A. The application of an induced bronchial collateral circulation to the coronary arteries by cardiopneumonopexy. II. Hemodynamics and the measurement of collateral flow to the myocardium. *Am. J. Path.*, 1956, **32**, 897-925.
6. Hanlon, C. R.; Sabiston, D. C., Jr., and Burke, D. R. Experimental pulmonary venous occlusion. *J. Thoracic Surg.*, 1952, **24**, 190-200.
7. Wyatt, J. P.; Burke, D. R., and Hanlon, C. R. Morphologic study of canine lungs after ligation of the pulmonary veins. *Am. J. Path.*, 1953, **29**, 291-303.
8. Hurwitz, A.; Calabresi, M.; Cooke, R. W., and Liebow, A. A. An experimental study of the venous collateral circulation of the lung. I. Anatomical observations. *Am. J. Path.*, 1954, **30**, 1085-1115.
9. Kety, S. S., and Schmidt, C. F. The determination of cerebral blood flow in man by the use of nitrous oxide in low concentrations. *Am. J. Physiol.*, 1945, **143**, 53-66.
10. Kety, S. S. Quantitative Determination of Cerebral Blood Flow in Man. In: *Methods in Medical Research*, Potter, V. R. (ed.). The Year Book Publishers, Inc., Chicago, 1948, **1**, 204-217.
11. Orcutt, F. S., and Waters, R. M. A method for the determination of cyclopropane, ethylene, and nitrous oxide in blood with the Van Slyke-Neill manometric apparatus. *J. Biol. Chem.*, 1937, **117**, 509-515.
12. Gregg, D. E.; Longino, F. H.; Green, P. A., and Czerwonka, L. J. A comparison of coronary flow determined by the nitrous oxide method and by a direct method using a rotometer. *Circulation*, 1951, **3**, 89-94.
13. Eckenhoff, J. E.; Hafkenschiel, J. H.; Harmel, M. H.; Goodale, W. T.; Lubin, M.; Bing, R. J., and Kety, S. S. Measurement of coronary blood flow by the nitrous oxide method. *Am. J. Physiol.*, 1948, **152**, 356-364.
14. Bing, R. J.; Hammond, M. M.; Handelsman, J. C.; Powers, S. R.; Spencer, F. C.; Eckenhoff, J. E.; Goodale, W. T.; Hafkenschiel, J. H., and Kety, S. S. The measurement of coronary blood flow, oxygen consumption, and efficiency of the left ventricle in man. *Am. Heart J.*, 1949, **38**, 1-24.
15. Liebow, A. A.; Hales, M. R.; Lindskog, G. E., and Bloomer, W. E. Plastic demonstrations of pulmonary pathology. *J. Tech. Methods*, 1947, **27**, 116-129.
16. Stern, H.; Ranzenhofer, E. R., and Liebow, A. A. Preparation of vinylite casts of the coronary vessels and cardiac chambers. *Lab. Investigation*, 1954, **3**, 337-347.

17. Liebow, A. A. The bronchopulmonary venous collateral circulation with special reference to emphysema. *Am. J. Path.*, 1953, **29**, 251-289.
18. Liebow, A. A.; Hales, M. R.; Bloomer, W. E.; Harrison, W., and Lindskog, G. E. Studies on the lung after ligation of the pulmonary artery. II. Anatomical changes. *Am. J. Path.*, 1950, **26**, 177-195.
19. Bruner, H. D., and Schmidt, C. F. Blood flow in the bronchial artery of the anesthetized dog. *Am. J. Physiol.*, 1947, **148**, 648-666.
20. Liebow, A. A.; Hales, M. R.; Harrison, W.; Bloomer, W. E., and Lindskog, G. E. The genesis and functional implications of collateral circulation of the lungs. *Yale J. Biol. & Med.*, 1950, **22**, 637-650.

[Illustrations follow]

LEGENDS FOR FIGURES

- FIG. 1. Dog 356. Proximal aortic angiogram, 2.1 seconds. The radio-opaque tip of the catheter is seen within the ascending aorta. Urokon has entered the coronary arteries and the left ventricle itself. The descending aorta and bronchial arteries are opacified. A posterior retrocardiac artery is visualized (R) extending from the circumflex branch of the coronary artery toward the bronchial arterial plexus, the mass of tortuous vessels lying anteriorly of the aorta. This posterior retrocardiac artery is demonstrated in the cast (Fig. 4).
- FIG. 2. Dog 356. Angiogram, 5.6 seconds. The pulmonary artery (P.A.) is visualized.
- FIG. 3. Dog 356. Angiogram, 10.6 seconds. The pulmonary vein (P.V.) has now become opacified. It lies anteriorly of the pulmonary artery. (For comparison with Fig. 2.)

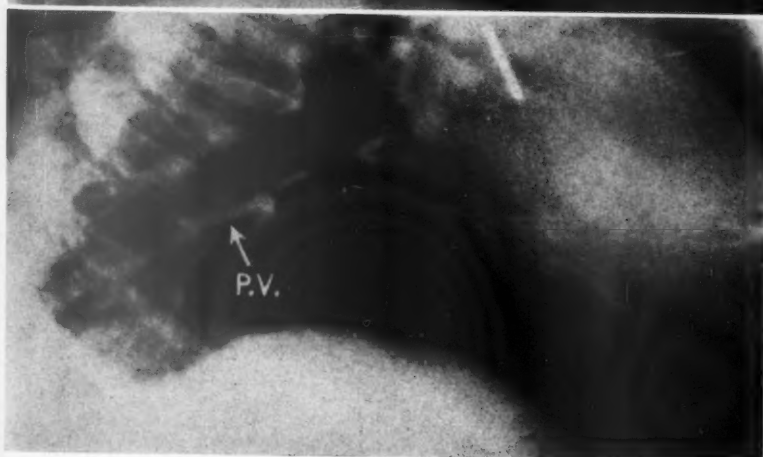
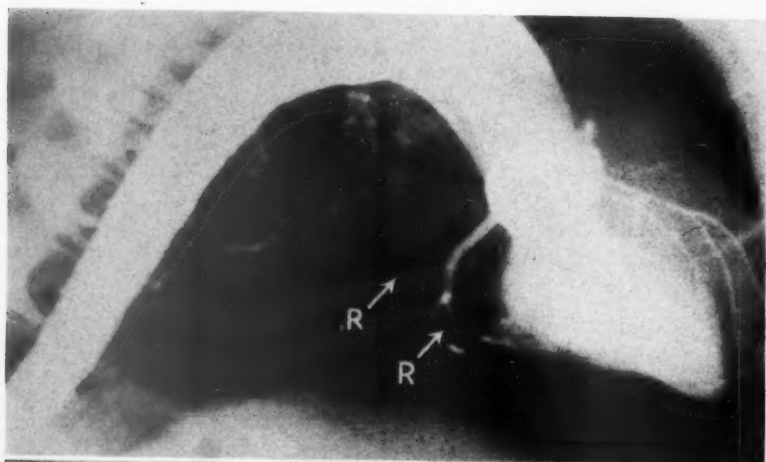


FIG. 4. Dog 356. Vinylite cast of cardiac and pulmonary structures, 17 $\frac{1}{4}$ months after operation. View of right aspect of heart and of medial aspect of left lung. The right lung has been removed. Two bronchial arteries (B.A.* 3 and 4) arise separately from the third right intercostal artery to be distributed to the subcarinal plexuses and thence to the lung. Each of these is accompanied by a large hilar bronchial vein (B.V. 1 and 2). For details see Appendix. A transpleural vein of diaphragmatic origin is indicated as B.V. 3. A posterior retrocardiac, the vessel demonstrated in the angiogram of Figure 1, is seen ascending from its origin in the circumflex along the posterior and lateral aspects of the left atrium (L.A.). Ao = aorta. Az = azygos vein. R.A. = right auricle.

* The numerical designations correspond to those used in the detailed protocols of the Appendix.

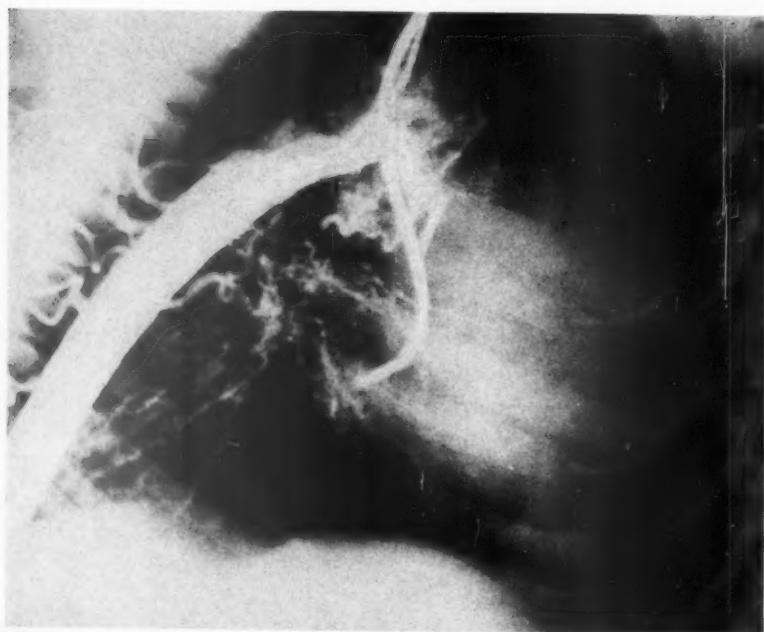


FIG. 5. Dog 358. Cast of cardiac and bronchial structures, 16 months after operation. Anterior view. Large transpleural collateral veins are seen, the most important (B.V. 2) descending from the internal mammary trunk of the superior vena cava (SVC). Small bronchial arteries (black) accompany branches of the transpleural veins (arrow). The pulmonary vein (P.V.) of the upper lobe has been injected retrogradely. It ends blindly at the hilum at the point of ligature. A hilar collateral vein (B.V. 1) extends from the azygos to the lung. A large retrocardiac artery (R) ascends on the anterior surface of the left atrium (L.A.) from its origin in the left circumflex to become continuous with a rich plexus of bronchial arteries that embraces the left upper lobe bronchus. Red plastic from the coronary and black plastic from the aorta are intermingled in this plexus. Other structures are labelled as in Figure 4.

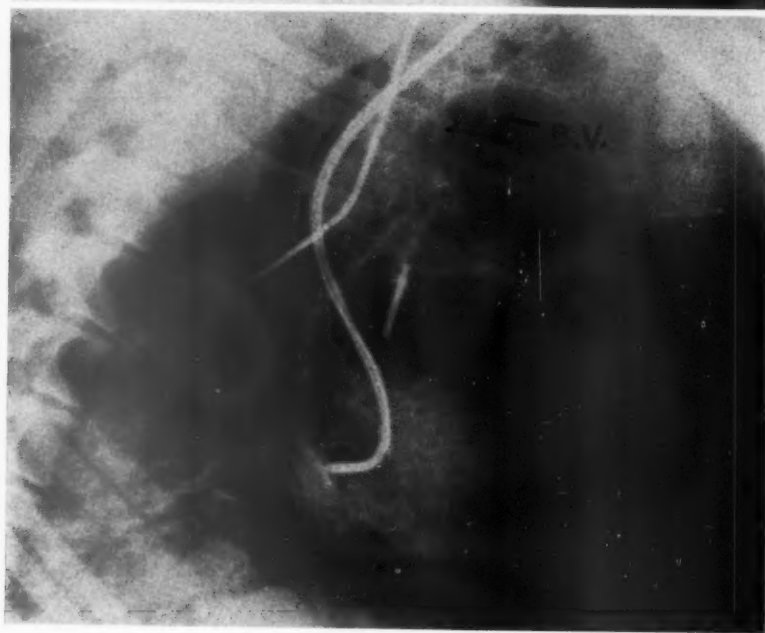


FIG. 6. Dog 361. Ascending thoracic angiogram, 2.1 seconds. The bronchial arteries are well visualized within the lung. The tip of the catheter that exhibits a sigmoid curve is within the coronary sinus.

FIG. 7. Dog 361. Angiogram, 6.3 seconds. Large tortuous opacified vessels (B.V.), interpreted as collateral veins, extend upward and forward from the opacified pulmonary vein. These tortuous vessels are considered to correspond to the collateral veins demonstrated in the cast (Fig. 8).



6



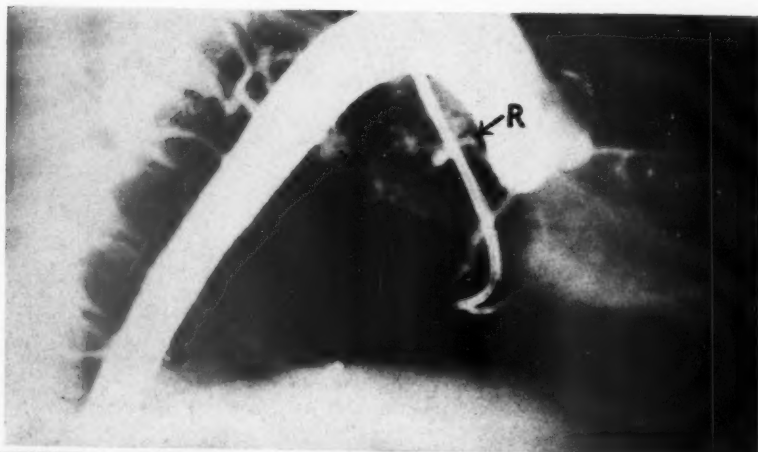
7

FIG. 8. Dog 361. Cast of cardiac and pulmonary structures, 16 months after operation. View corresponds to Figure 4. Two large transpleural collateral veins correspond to those demonstrated in the angiogram of Figure 7. B.V. 3 drains into the left vertebral vein, and B.V. 4 is tributary to the internal mammary venous trunk. A hilar bronchial vein is shown also (B.V. 2). Legend otherwise as in Figure 4.



FIG. 9. Dog 360. Proximal aortic angiogram, 1.4 seconds. A large retrocardiac artery (R) is seen to extend from the circumflex to become continuous with the bronchial arterial plexus. For comparison with the cast shown in Figure 11.

FIG. 10. Dog 360. Angiogram, 2.8 seconds. There is now much more complete filling of the bronchial arterial plexus. The vessel in the position of the retrocardiac (R) is still visible, but less plainly than in Figure 9. The sharp, bar-shaped shadow in the region of the diaphragm, anteriorly of the aorta, is the radio-opaque end of the catheter in the inferior vena cava. The posteriorly arched tip of the catheter in the coronary sinus can also be seen, as in Figure 9.

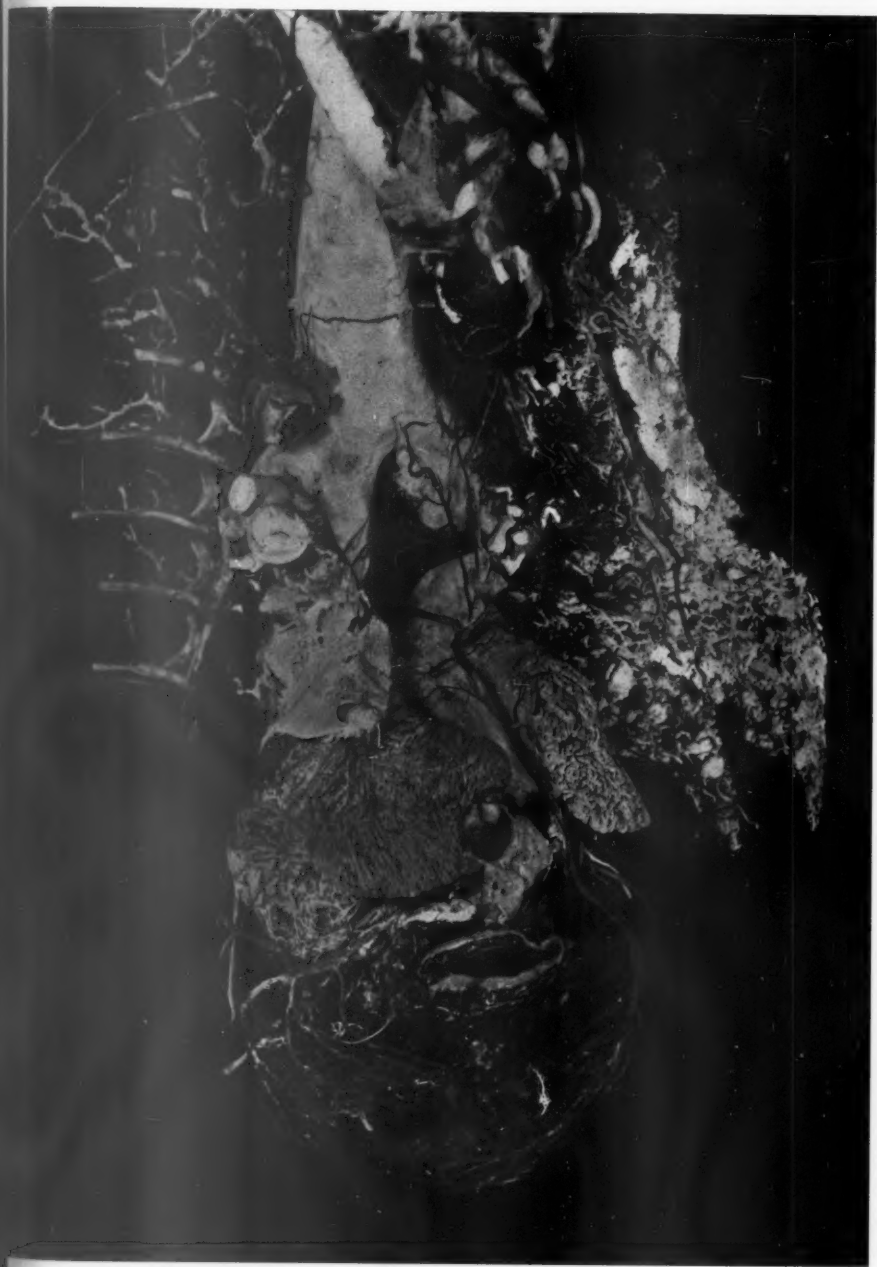


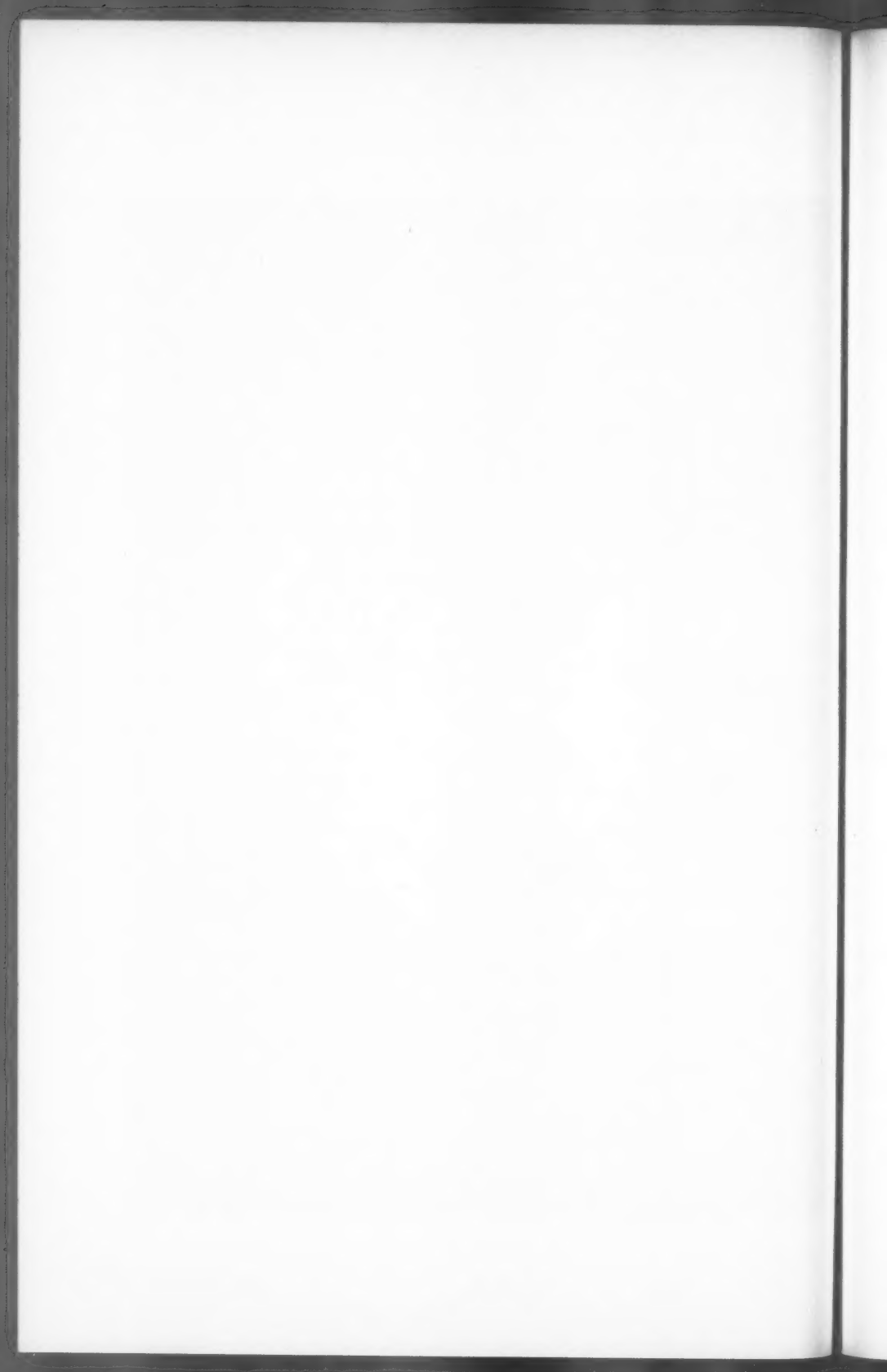
9



10

FIG. 11. Dog 360. Cast of heart and pulmonary structures 16 months after operation. The heart and anteromedial surface of the left lung are shown. The right lung has been removed. The large retrocardiac artery, demonstrated in the angiograms of Figures 9 and 10, is seen to have been injected with red plastic from the left circumflex coronary artery. It ascends along the anterior surface of the left atrium at the base of the auricular appendage (green), to become continuous with a very extensive plexus of bronchial arteries on the mediastinal aspect of the left lung. This plexus also has received from the aorta some black plastic which has become commingled with the red. Elements of the transpleural venous plexus derived from the left vertebral vein can be seen injected in yellow on the medial aspect of the left lung. Bronchial arteries accompany some of the smaller branches of the collateral veins. More deeply situated, can be seen several large pulmonary venous trunks injected retrogradely in yellow from the transpleural collateral veins, and from hilar collaterals. The azygos vein, superior vena cava, and aorta are shown also, as in Figure 5.





FIFTY-FOURTH ANNUAL MEETING
OF THE
AMERICAN ASSOCIATION OF PATHOLOGISTS
AND BACTERIOLOGISTS
WASHINGTON, D.C.
APRIL 11TH, 12TH, AND 13TH, 1957



THE AMERICAN ASSOCIATION OF PATHOLOGISTS
AND BACTERIOLOGISTS

Fifty-fourth Annual Meeting

HOTEL STATLER

Washington, D.C.

April 11th, 12th, and 13th, 1957

PRESIDENT BENNETT IN THE CHAIR

BUSINESS MEETING

April 11, 1957

The following nominations for elective officers were submitted by the Council:

<i>President</i>	DR. SIDNEY FARBER
<i>Vice-President</i>	DR. ALAN R. MORITZ
<i>Secretary</i>	DR. RUSSELL L. HOLMAN
<i>Treasurer</i>	BRIG. GEN. ELBERT DECOURSEY
<i>Incoming Member of Council</i>	DR. D. MURRAY ANGEVINE

Additional nominations were sought from the floor. None having been offered, it was moved, seconded, and directed that the Secretary be instructed to cast a unanimous ballot for the entire slate.

The President announced that the Council had elected Dr. J. Earle Ash by acclamation to complete the term of Dr. G. Lyman Duff, deceased, as member of the Council.

At the direction of the President, the Secretary reported the following actions of the Council:

Election of New Members

Adriano, Salvador M.	Fennell, Robert H., Jr.
Arean, Victor M.	Hennigar, Gordon R., Jr.
Bahn, Robert C.	Hilberg, Albert W.
Bauer, Heinz	Hoch-Ligeti, Cornelia
Bowden, Drummond H.	Jones, Thomas C.
Brunson, Joel G.	Leighton, Joseph
Carlson, Arthur S.	Luse, Sarah A.
Coon, Robert W.	McAdams, George B.
Cox, Thomas R., Jr.	Meissner, George F.
Cronin, M. T. I.	Newman, William

THE AMERICAN ASSOCIATION OF PATHOLOGISTS
AND BACTERIOLOGISTS

Fifty-fourth Annual Meeting

HOTEL STATLER

Washington, D.C.

April 11th, 12th, and 13th, 1957

PRESIDENT BENNETT IN THE CHAIR

BUSINESS MEETING

April 11, 1957

The following nominations for elective officers were submitted by the Council:

<i>President</i>	DR. SIDNEY FARBER
<i>Vice-President</i>	DR. ALAN R. MORITZ
<i>Secretary</i>	DR. RUSSELL L. HOLMAN
<i>Treasurer</i>	BRIJ. GEN. ELBERT DECOURSEY
<i>Incoming Member of Council</i>	DR. D. MURRAY ANGEVINE

Additional nominations were sought from the floor. None having been offered, it was moved, seconded, and directed that the Secretary be instructed to cast a unanimous ballot for the entire slate.

The President announced that the Council had elected Dr. J. Earle Ash by acclamation to complete the term of Dr. G. Lyman Duff, deceased, as member of the Council.

At the direction of the President, the Secretary reported the following actions of the Council:

Election of New Members

Adriano, Salvador M.	Fennell, Robert H., Jr.
Arean, Victor M.	Hennigar, Gordon R., Jr.
Bahn, Robert C.	Hilberg, Albert W.
Bauer, Heinz	Hoch-Ligeti, Cornelia
Bowden, Drummond H.	Jones, Thomas C.
Brunson, Joel G.	Leighton, Joseph
Carlson, Arthur S.	Luse, Sarah A.
Coon, Robert W.	McAdams, George B.
Cox, Thomas R., Jr.	Meissner, George F.
Cronin, M. T. I.	Newman, William

Norman, Thomas D.
Odessky, Louis
Olson, Carl
Ortega, Louis G.
Roberts, James C., Jr.

Stamler, Frederic W.
Stowens, Daniel
Thomas, Wilbur A.
Totten, Robert S.
Wexler, Bernard C.

With deep regret the deaths of the following members were recorded:

Austrian, Charles R.
Bartlett, Charles J.
Bauer, William H.
Duff, G. Lyman
Durlacher, Stanley H.
Funke, John

Gonzales, Thomas A.
Medlar, Edgar M.
Russell, Nelson G.
Saxton, John A.
Spitz, Sophie
Wahl, Henry R.

Weller, Carl V.

The Secretary announced that the next annual meeting of the Association will be held in Cleveland, Ohio, on April 24, 25, and 26, 1958. The topic for the symposium is "Natural and Acquired Factors in Resistance to Disease." The referee will be Dr. Louis T. Pillemer.

The Secretary further announced that the annual meeting of the Association in 1959 will be held in Boston, Massachusetts.

The President requested that the Acting Editor-in-Chief of *The American Journal of Pathology*, Dr. Ernest W. Goodpasture, acquaint the members of the Association with problems pertaining to the Journal. Dr. Goodpasture related in detail the results of a broad survey of future financial requirements.

The President expressed the warm appreciation of the Council and of the Association for Dr. Goodpasture's ready assumption of the duties of Acting Editor-in-Chief upon the death of Dr. Carl V. Weller and of his wise and effective administration of the affairs of the Journal.

The President further expressed the deep gratitude of the Council to Miss Dorothy E. Seiferlein, Editorial Assistant of *The American Journal of Pathology*, for her loyal and devoted service, and to Dr. A. James French, to Dr. Murray R. Abell, and to the authorities of the School of Medicine, University of Michigan, for their patient cooperation in relation to Journal activities in Ann Arbor.

The President announced that the Council, having considered the advice of the Acting Editor-in-Chief and the growing financial responsibilities of the Journal, has directed that membership dues in the Association be increased to \$15.00 per annum effective January 1,

1958. This constitutes the only dues increment since the inception of the Association in 1902.

It was further announced that the subscription rate for *The American Journal of Pathology* to non-members would also be increased to \$15.00 per annum effective January 1, 1958.

The President announced the appointment of Dr. Edward A. Gall as Editor-in-Chief of *The American Journal of Pathology*.

Dr. Alan R. Moritz, member of the Council, advised the members of the suggestion received by the Council that the annual meeting of the Association might be more conveniently held in February rather than April, as has been customary. A show of hands indicated no clear majority favoring such a change.

The President directed the Secretary to read Amendments to the By Laws of the Association approved by Council.

It is recommended that:

"By Law 6: The Constitution and By Laws may be amended by a vote of the Association at a meeting subsequent to that at which such amendment was proposed, by a vote of three fourths of the members present."

become "By Law 7"

It is recommended that:

"By Law 6: (a) Members in good standing who, by reason of age or physical disability, have retired from gainful professional activity may, upon application to Council, be granted Emeritus Membership. (b) Emeritus Members shall remain upon the rolls of the Association and shall receive regular notices. They shall, however, be relieved of payment of dues and may neither hold office nor receive *The American Journal of Pathology* except by independent subscription."

be adopted.

No action upon these amendments being required until the next annual meeting, no discussion ensued.

The President then asked if there was any business from the floor. There being none, the business meeting adjourned.

Edward A. Gall, *Secretary*

REPORT OF THE TREASURER

The report of the Treasurer was submitted to the Council and accepted. It was accompanied by a letter of certification from Ralph

Cole, Certified Public Accountant, of Washington, D.C. In condensed form, the Treasurer's report follows:

General Checking Account

Receipts

Balance on hand, January 1, 1956.....	\$ 6,700.56
Membership dues	\$9,250.10
Interest on bonds and savings accounts	874.35
Income from annual meeting	336.00
	<hr/>
	10,460.45
Total receipts	<hr/>
	\$17,161.01

Disbursements

American Journal of Pathology	\$7,376.00
Secretary's office, clerical	\$ 425.00
Printing, supplies, miscellaneous	1,049.57
	<hr/>
	1,474.57
Treasurer's office, bonding and auditing.....	155.00
Printing, supplies, miscellaneous	96.85
	<hr/>
	251.85
Miscellaneous, annual meeting.....	133.54
International Academy of Pathology.....	139.77
Intersociety Committee for Research	
Potential in Pathology.....	100.00
	<hr/>
	373.31
Total disbursements	<hr/>
	\$ 9,475.73
Balance on hand, December 31, 1956.....	<hr/>
	\$ 7,685.28

Investment Inventory

U.S. Treasury Bonds, series G	\$20,000.00
Savings accounts	
Riggs National Bank	\$1,254.47
National Bank of Washington	3,591.87
Olympic Savings & Loan Association, Berwyn, Ill.	5,000.00
Home Savings & Loan Association, Los Angeles	5,000.00
	<hr/>
	14,846.34
Earned but unrealized interest on U.S. bonds	250.00
	<hr/>
Total of investment inventory	<hr/>
	\$35,096.34

Elbert DeCoursey, Treasurer

SCIENTIFIC PROCEEDINGS

ABSTRACTS

THE EFFECT OF STAPHYLOCOCCAL TOXIN ON THE PULMONARY TISSUES OF THE RABBIT. J. R. Jackson, R. J. Gibbons, and D. Magner,* University of Ottawa Faculty of Medicine, Ottawa, Ont.

The introduction of staphylococcal toxin into the bronchial tree of adult rabbits produced widespread hemorrhagic necrosis of the lungs and inflammatory lesions closely resembling those seen in primary staphylococcal pneumonia in infancy.

Early lesions consisted of degenerative changes in bronchial epithelium and vascular endothelium. Dilatation of capillaries and pulmonary edema ensued. Rupture of capillaries and small vessels was frequent, producing grossly visible hemorrhagic lesions within 1 hour and 45 minutes. Later, larger blood vessels became dilated and some showed thrombosis. A profuse polymorphonuclear leukocytic exudate began in these areas within 4 hours. Within 24 hours, there were large areas of hemorrhagic necrosis, with peripherally situated suppurative zones. In some animals small sterile micro-abscesses developed.

Administration of the toxin followed shortly by the injection of a solution of trypan blue into the inferior vena cava sometimes resulted in multiple, scattered, small, peripheral, non-staining areas in the lungs. These were thought to indicate initial pulmonary vascular spasm. When the dye was injected later in animals showing hemorrhagic lesions, the areas of exclusion of the dye were much larger, occupying a lobe or the whole lung. This was attributed to vascular paralysis with circulatory stasis. It is possible that a similar mechanism may account for the early cyanosis and rapidly fatal outcome in some cases of staphylococcal pneumonia in infants.

EXPERIMENTAL STUDIES IN INTRAVASCULAR THROMBOSIS AND PULMONARY EMBOLISM. David G. Freiman* and Stanford Wessler, Beth Israel Hospital and Harvard Medical School, Boston, Mass.

It is well established that serum or fractions thereof will accelerate coagulation *in vitro* and will induce thrombosis in animals. The systemic infusion of heterologous canine serum induces in dogs a transient hypercoagulable state during which massive thrombosis develops in venous segments containing stagnant blood far removed from the site of infusion. Such thrombi, when fresh, may exhibit many of the gross and microscopic features of postmortem clot, but are easily recognized when heparin is administered preterminally to prevent agonal or postmortem coagulation. With this method it is possible to produce thrombi of different size and age in peripheral veins without the introduction of foreign substances and to observe the behavior of these thrombi after their release to the lungs. When single, small, fresh clots 4 cm. in length and 10 minutes in age were released, no alterations in hemodynamics were observed. Pulmonary emboli were found in all animals sacrificed up to the 4th day, in half the animals sacrificed between the 4th and 13th days, and in no animals sacrificed between the 14th and 28th days after embolization. Clot fragmentation was frequent and significant reductions in total volume of clot were uniformly noted within 24 hours. Clot adherence was found only after the 5th day and no evidence of pulmonary infarction was observed.

* Asterisks indicate members of The American Association of Pathologists and Bacteriologists. All others appear on the program "by invitation."

Similarly, when massive amounts of fresh clot up to 200 cm. in total length were released to the pulmonary circulation 10 minutes after formation, dramatic reductions in volume were noted within 24 hours; after several days only small fragments, partially adherent, could be identified. In this latter group pulmonary infarcts were noted occasionally, but their small size was out of all proportion to the total volume of clot released. When, however, small clots 4 cm. in length were allowed to remain *in situ* in a peripheral vein for 2 weeks before being released, the embolus recovered in the lung after 2 to 4 additional weeks showed little evidence of fragmentation and minimal or no reduction in total volume. These findings demonstrate the remarkably efficient mechanisms that exist for the rapid disposal of fresh thrombi produced in this fashion. On the other hand, the efficiency of these mechanisms appears to be markedly impaired when aged and partially organized clots are released. The observations reported herein may account for some of the discrepancies encountered in the clinical and pathologic diagnosis of pulmonary embolism in man.

EXPERIMENTAL PULMONARY EMBOLISM IN DOGS. William E. Jaques* and Albert L. Hyman, Louisiana State University School of Medicine, New Orleans, La.

Nineteen mongrel dogs received weekly intravenous injections of autogenous clots for periods up to 10 weeks. Eight of these dogs had ligation of an azygos vein previous to the introduction of emboli in an attempt to study the effect of shunts. Five dogs had cardiac catheterizations performed at various stages during the injections. Electrocardiograms were taken during the injection of the clots in all animals.

There was a sharp and pronounced rise in pulmonary arterial pressure accompanied by an increase in pulmonary arterial resistance following the introduction of emboli. The elevated pressures were not sustained initially but with repeated injections of autogenous clots, a sustained elevation of pulmonary arterial pressure was manifested. Changes in the electrocardiogram and the effect of atropine led to the belief that vagus stimulation played an important rôle in the development of pulmonary arterial hypertension. No appreciable difference in pressure tracings was noticed between the animals with and without the obliteration of shunts.

The histologic changes revealed stages of organizing emboli with eventual eccentric intimal thickening. The early basophilic change, elastic tissue proliferation, growth of smooth muscle cells, and collagenization will be described. Examples of necrotizing arteritis and arteriolitis were noted.

AN INFLUENCE OF FETAL DIFFERENTIATION ON CARCINOGENESIS. Stanfield Rogers,* Duke University School of Medicine, Durham, N.C.

It was determined to find whether the gradual qualitative and quantitative changes associated with the differentiation and maturation of fetal mouse lung would influence the number or class of tumors initiated by a single brief exposure to ultraviolet irradiation. The period of gestation of mice is 19 to 20 days. Lung tissue from fetal mice varying in age of gestation from 10 to 18 days was excised, hashed into tiny fragments, exposed to ultraviolet irradiation under a film of Ringer's solution at 37° C. for 15 minutes, and implanted in the leg muscles of mice of the same strain (A). After an interval of at least 2 months the implants were excised, cut in serial section, and examined microscopically for the presence of tumors. The tissues from mice irradiated at 18 days' gestation developed, almost exclusively, adenomas of the alveolar cell variety. The tissues from animals of 14 and 15 days' gestation yielded tumors wholly of the bronchial type. The alveolar sacs are just beginning to be formed at this time. The absence of the initiation of alveolar cell adenomas in tissue of this age of gestation appears directly related to the relative absence of cells of this type at the time of irradiation. No tumors

have been found in the tissues irradiated at a gestation age of 13 days or less. It is possible that this is, at least to some degree, related to the relatively small number of cells in the lungs at this time.

These findings make plain that when a tumor is initiated in cells with the capacity to develop into cells of other types (bronchial bud to alveolar cells), the tumor is of the type to which the carcinogenic stimulus is applied. They also provide evidence indicating that the pathogenesis of the neoplastic change initiated by urethane and ultraviolet irradiation takes place through wholly different pathways even in initiating tumors of the same morphologic type. It is known that urethane exposure of fetal mice of as little as 14 days' gestation will initiate adenomas of the alveolar cell type. This is in contrast to the effects of ultraviolet irradiation.

EPITHELIAL CHANGES OF THE RESPIRATORY TRACT IN RELATION TO THE SITES OF PARTICLE DEPOSITION IN EXPERIMENTAL SPECIES. Paul Kotin* and Charles J. McCammon, University of Southern California School of Medicine, Los Angeles, Calif.

Particle size and configuration in addition to the chemical nature of environmental agents are significant factors in determining the effect of inhalants on the respiratory tract in experimental species and probably in man. Their effect has been demonstrated in studies on tumor induction in the skin and subcutaneous tissues. We have been concerned with (1) the effect of these factors on the pattern of particulate deposition and retention in the tracheobronchial tree and (2) the epithelial changes that follow. We are reporting the findings observed following the exposure of mice and rats to an atmosphere of ozonized gasoline and rabbits following exposure to soot.

Two strains of mice with contrasting rates of spontaneous pulmonary tumors (A strain and C57 black) and an inbred strain of rats (Manor Park) were exposed in inhalation chambers to an atmosphere of ozonized gasoline rich in the oxidation products of chain and branched aliphatic hydrocarbons. Control animals were exposed in inhalation chambers containing washed air.

In mice of the A strain, significant variations in incidence of pulmonary tumors were noted between the test and control animals. In C57 black mice, the over-all rate of tumor induction in the test mice was greater than in the controls. Of note was the finding that the spontaneous tumor rate in the C57 black control mice was significantly higher than that recorded by others in earlier investigations. Differences in the natural history of tumors were observed between the test and control mice.

Pulmonary epithelial changes were observed in the rats under study. In this species, hyperplastic and metaplastic changes were frequent and marked. Papillary overgrowth of the bronchiolar epithelium with focal areas of atypical change was observed. Detailed study of the epithelial changes in the test and control rats revealed marked histologic differences. These will be discussed.

Rats and rabbits were exposed to different atmospheres containing soot particles varying in size from 180 Å to 0.5 μ in diameter. Exposures were made both to atmospheres containing particles of uniform size and atmospheres containing a variety of particle sizes. Animal species were either exposed to irritants prior to soot exposure or to soot alone. Prior exposure to irritants, as for example smog, resulted in abnormal precipitation and retention of particles on the bronchial and bronchiolar epithelium. The pattern of deposition and the exaggerated retention sites of particulate accumulation were correlatable with the time and intensity of prior irritant exposure. They further appeared to be responsible for the histologic changes seen at deposition sites. The significance of particulate deposition, retention, and epithelial changes in relation to pulmonary carcinogenesis will be discussed.

ACINAR TYPE OF ATYPICAL PROLIFERATION IN THE LUNG IN RELATION TO THE CANALS OF LAMBERT. Herman M. Brandt and Averill A. Liebow,* Yale University School of Medicine, New Haven, Conn.

In 1955, Lambert described channels in the walls of normal bronchioles that connect their lumina directly with adjacent alveoli. In the presence of chronic pulmonary disease such as emphysema and organizing pneumonitis, especially in older individuals, these canals become enlarged and lined by columnar, cuboidal, or squamous epithelium continuous with that of the bronchiole. Epithelium-lined labyrinthine spaces of varying degrees of complexity are thus formed, often associated with "emphysema," to which several bronchioles may contribute. These acinar structures vary in size, depending upon forces of destruction or traction that expand the distal air spaces. To some of these lesions, the gross descriptive term honeycomb lung has been applied.

These appearances often have been misinterpreted as "congenital cystic disease" of the lung. In some instances, the lining epithelium may become both hyperplastic and anaplastic, so that distinction from "bronchiolar carcinoma" ("pulmonary adenomatosis") may be difficult. It is possible that some metastasizing peripheral acinar tumors of the lung so classified may have their origin in such initially regenerative processes of varying etiology.

THE ASSOCIATION OF TERMINAL BRONCHIOLAR CARCINOMA (ALVEOLAR CELL TYPE) WITH DIFFUSE INTERSTITIAL PULMONARY FIBROSIS. David M. Spain,* Beth-El Hospital, Brooklyn, N.Y.

Analysis of cases of terminal bronchiolar carcinoma (alveolar cell type) indicates a frequent association with diffuse interstitial inflammation and fibrosis of the lungs. Regenerative hyperplasia of alveolar lining cells is common in these chronic and progressive pulmonary conditions. At times areas appear adenomatous. Transition forms can be seen leading directly into carcinoma. The diffuse interstitial fibrosis of the lungs appears to antedate the carcinoma. The origin of these lesions seems to indicate multicentricity rather than unicentricity. The origin of the pulmonary fibrosis in most instances is not clear; it presumably may be related to any number of atmospheric pollutants.

PULMONARY CARCINOMA: CORRELATION BETWEEN MORPHOLOGY AND SURVIVAL. Fred C. D. Collier,* Robert H. Kyle, H. T. Enterline, Theodore Tristan, and Roy Greening, University of Pennsylvania School of Medicine and the Presbyterian Hospital, Philadelphia, Pa., and University of Rochester School of Medicine, Rochester, N.Y.

A combined morphologic, histochemical, and biologic survey was used in re-evaluation of all patients with excision of primary carcinoma of the lung performed at the Hospital of the University of Pennsylvania in the 16 year period ending January 1, 1956. One hundred per cent follow-up was obtained on all of these patients. Every effort was made to establish the histogenesis of tumors, even though some were composite in type and, as will be shown, tumors were classified as undifferentiated only after exhaustive studies failed to delineate their probable origin. Following the suggestion of Warren, selected sections were stained for elastica to elucidate blood vessel invasion. The necessity of elastic stains will be appropriately stressed. The study emphasizes the importance of recognition by the surgical pathologist of two concepts if his reports are to serve the thoracic surgeon as prognostic indicators.

The first of these concepts is in regard to the prognostic importance of vascular invasion by pulmonary carcinoma. Surprisingly frequent, vascular invasion was found in over two thirds of lungs resected for cancer. The prognosis for this group

must, of necessity, be uniformly poor for only 3 per cent of patients with pulmonary carcinoma showing vascular invasion survived 5 years.

The second concept, enabling the pathologist to predict relative benign course, concerns the definition of the term localized. The surgical meaning of the term does not include the pathologist's findings but refers only to observations at thoracotomy. Limiting the term localized to apply only to those tumors which, regardless of size or location at operation, showed no extension of surrounding structures and of which morphologic study showed no invasion of blood vessels or lymph nodes, the authors found an 89 per cent 5 year survival.

It will be shown that the generally grave prognosis accorded patients with lung cancer is a reflection of vascular invasion and that those tumors which have extended to regional lymph nodes do not always have the biologic potential for vascular invasion. Morphologically identical tumors of all types will be shown, some with vascular invasion with its attendant poor prognosis, others with prolonged survival showing no vascular invasion. The factor of host resistance and the antagonistic factor of tumor potential will be discussed.

CARCINOGENESIS IN THE CERVIX UTERI OF THE MOUSE; THE EARLY EPITHELIAL CHANGES. James W. Reagan* and W. Budd Wentz, Western Reserve University School of Medicine and University Hospitals, Cleveland, Ohio.

In recent years, considerable emphasis has been placed on the early detection of cancer in the uterine cervix as well as in other sites. As a result, many so-called precancerous lesions are detected and treated even though it has not been established conclusively that such a phase is always represented in carcinogenesis nor is it known whether the lesions in question represent irreversible stages in the developmental process.

This study is concerned with the epithelial changes seen early in experimentally induced cancer of the cervix uteri of the mouse. As in previous studies cancer was induced by suspending a carcinogen-impregnated thread in the cervical canal. Cellular studies of vaginal washings were utilized in order to follow carcinogenesis without disturbing the developing lesion and to establish a cyto-histopathologic correlation.

The purpose of this presentation is to describe the epithelial changes occurring early in carcinogenesis; to indicate the frequency with which these changes are observed in carcinogenesis; and to consider whether the lesions represent an irreversible phase of carcinogenesis.

EFFECT OF PROLONGED ADMINISTRATION OF SPERMICIDAL CONTRACEPTIVES ON RATS KEPT ON LOW PROTEIN OR ON FULL DIET. Cornelia Hoch-Ligeti, University of Virginia Medical School, Charlottesville, Va.

Two spermicidal contraceptives were investigated for carcinogenic activity in rats. The contraceptives were administered either by vaginal instillation or by oral feeding to rats kept on a full diet or on a purified diet which had a low-protein content.

In the 14 rats fed the full diet and treated with the contraceptives the number of tumors did not exceed the number expected in aging rats. The 39 rats kept on the low-protein diet developed liver damage and 13 had mammary tumors. One uterine and one intestinal tumor were observed.

In two separate experiments, 73 of 95 rats kept on the low-protein diet and treated with contraceptives, either by vaginal or oral administration, developed tumors. Apart from mammary and hepatic tumors, which occurred also in untreated rats kept on the same diet, 69 tumors in other locations occurred. The large number and unusual localization of the tumors (e.g., two in the brain) render the probability of the results being due to chance extremely small.

The chemical substance responsible for the tumor production is not yet known. The carcinogenic activity of one constituent of contraceptive A, 8-ortho hydroxyquinoline, is now under investigation. The implication of the findings relative to the problem of cervical carcinoma in humans will be discussed.

A TOPOGRAPHIC STUDY OF THE ATYPICAL EPITHELIAL CHANGE OF THE UTERUS ASSOCIATED WITH THE PRESENCE OF CHORIONIC TISSUE; DEMONSTRATION OF THE ALTERATION IN THE ENDOCERVIX. Javier Arias-Stella,* Faculty of Medicine, Lima, Peru.

Recently an atypical endometrial change occurring in cases of uterine abortion, hydatidiform mole, chorio-epithelioma, syncytial endometritis, and ectopic pregnancy has been described. The change is characterized by nuclear enlargement of isolated cells, proliferation of cells, and loss of cellular polarity. The involved glands are proliferative or secretory and may show both pathologic activities simultaneously. The alteration tends to be focal. When one is not familiar with this atypical change, the question of malignant tumor may be raised on histologic grounds. Instances of the change in the past had been interpreted as carcinoma. The fact that this alteration seems independent of the decidual reaction calls for a further investigation of placenta-endometrium interrelationships.

My early observations of the change were upon material from which conclusions concerning its distribution and extent could not be drawn.

Five cases of chorio-epithelioma and one of hydatidiform mole have been studied by serial blocking. From 60 to 120 blocks were made from each uterus. These included the entirety of the endometrial, endocervical, and exocervical surfaces. Each block was labeled and thus a mapping of the areas showing the change was made. In four cases of chorio-epithelioma and in the one of mole, the salient alteration was present. In the other case of chorio-epithelioma it was absent.

The change occurred focally, apparently having a random distribution. It could extend over 30 to 50 per cent of the endometrial surface, or be limited to only a few foci. The location was not related to the areas of chorionic implantation. In two cases the alteration was seen also in the endocervix. Here the nuclear enlargement, cell proliferation, and loss of polarity were identical with the changes in the endometrium.

FUNCTIONING STROMA-STIMULATING OVARIAN TUMORS. Robert E. Scully,* Massachusetts General Hospital, Boston, Mass.

Certain ovarian tumors generally considered to have no endocrine significance are now and then accompanied by clinical changes suggesting function. Several authors have reported instances of the association of cystadenomas, Brenner tumors, or Krukenberg tumors with endometrial hyperplasia. Others have pointed out the occasional presence of lutein-like cells or of fat in the stroma of these tumors. Only a few investigators, however, have tried to correlate morphologic changes in the neoplastic stroma with clinical evidence of endocrine imbalance. Three cases are presented which strongly suggest the presence of functioning stroma in an ovarian tumor.

The first patient was a 68-year-old woman who bled from a hyperplastic endometrium. The right ovary was atrophic; the left was occupied by a 5 cm. cystic Brenner tumor. Its stroma contained plump cells suggesting theca externa as well as round clear elements resembling theca lutein cells.

The second patient was a 74-year-old woman who bled from a hyperplastic endometrium. Other findings were the presence of secretion in the breasts and a marked enlargement of the clitoris. One ovary was atrophic; the other was replaced by a 9 cm. multilocular serous cystadenofibroma. Within the latter was a 2 cm. nodule of adenocarcinoma. The stroma of the malignant portion contained

numerous nests of cells resembling theca lutein cells. Although the clitoris remained large, the breast secretion was noted to have disappeared 2 months after surgical removal.

The third patient was a 24-year-old woman who experienced progressive hirsutism and enlargement of the clitoris. Exploration revealed a slightly enlarged polycystic left ovary. The right ovary, containing a 3.5 cm. Brenner tumor, was removed. The stroma of the tumor was composed of plump cells containing fat. It harbored nests of cells indistinguishable from theca lutein or Leydig cells. There was no regression of the virilism postoperatively. However, the remarkable coincidence of progressive virilism with an enlarging ovarian tumor containing this unusual stroma suggests that (1) the tumor stroma was producing an androgen or (2) the opposite ovary was virilizing the patient, and the tumor stroma, reacting to whatever was stimulating the opposite ovary, might have been contributing to the virilism. A normal 17-ketosteroid excretion was evidence against adrenal hyperfunction.

In a few cases, histochemical studies have been done on tumors in this category and have shown staining reactions similar to those seen in thecoma. Since only about 5 per cent of primary tumors of the ovarian stroma are functioning thecomas and the remainder are non-functioning fibromas, the fact that only a small proportion of tumors that stimulate ovarian stromal proliferation are accompanied by endocrine imbalance does not exclude the possibility of a cause and effect relationship between tumor and endocrine change. More experience is needed with this type of tumor before it takes its final place among functioning ovarian tumors.

POLYEMBRYONIC EMBRYOMA OF THE OVARY OF PARTHENOGENETIC ORIGIN. L. C. Simard,* Notre-Dame Hospital, Montreal, Que.

An embryoma of the ovary containing innumerable embryonic formations similar to the structure of early human embryos is presented. A short review is offered of the facts favoring the parthenogenetic origin of embryomas of the gonads.

THE HISTOGENESIS OF LICHEN SCLEROSUS ET ATROPHICUS AND ITS RELATIONSHIP TO KRAUROSIS VULVAE. Wallace H. Clark, Jr.,* Tulane University School of Medicine, Charity Hospital of New Orleans, and the Ochsner Foundation Hospital, New Orleans, La.

Lichen sclerosus et atrophicus is an uncommon disorder of the skin of relatively little clinical significance. Recently, it has aroused interest because it has been emphasized that a histologically similar disorder frequently involves the vulva.

The present studies suggest that many cases clinically classified as kraurosis vulvae have the histologic changes of lichen sclerosus et atrophicus, and some of them show many extragenital lesions of the latter condition. Histologically, one of the earlier microscopic changes in the extragenital and genital lesions is subepidermal edema which may be severe and limited to the papillary corium. The edema forces the epidermis away from the underlying corium and is associated with atrophy of the epidermis. Following this there is gradual deposition of dense connective tissue in the edematous papillary corium. Subsequently, edema may again separate the epidermis from the now sclerotic corium, ultimately resulting in further fibrosis. The underlying reticular corium usually shows a zone adjacent to the area of subepidermal fibrosis which is infiltrated by lymphocytes and histiocytes.

The process, therefore, would appear to be the continual formation of subepidermal edematous blebs followed by gradual fibrosis. These lesions may become confluent on the vulva, resulting in the sclerotic process observed clinically. The extragenital lesions usually remain discrete, but the changes in both sites are virtually identical morphologically. The studies carried out thus far have not

shown atypical epithelial changes that would warrant the classification of these lesions as histologically premalignant.

ELECTRON MICROSCOPY AND BIOCHEMISTRY OF WALLERIAN DEGENERATION IN THE OPTIC AND TIBIAL NERVES. Sarah A. Luse and Richard E. McCaman, Washington University School of Medicine, St. Louis, Mo.

Wallerian degeneration of the optic and tibial nerves of the rabbit was studied at 15, 45, 100, and 200 days after enucleation of the eye and section of the tibial nerve. A small piece of each nerve was fixed for electron microscopy. The remainder of the nerves as well as nerves from other animals similarly treated were taken for light microscopy or chemical and enzymatic studies. The tissues for chemical studies, frozen in liquid nitrogen, were sectioned in a cryostat refrigerated at -20°C . and lyophilized.

Chemical determinations of lipid content revealed a striking difference between a degenerating central tract (optic nerve) and degenerating peripheral nerve (Rossiter). In the optic nerve there was no lipid loss at 15 days following section, slight loss at 45 days, and only at 100 days was the lipid decrease severe. In the peripheral nerve lipid has been shown to be markedly decreased by 16 days and by 100 days only a scant amount remains. These findings suggested that phagocytosis would be a later occurrence in the optic nerve than in the tibial nerve, which was corroborated by the electron micrographic observations.

In optic nerves examined by electron microscopy, degeneration of axons was evident at 15 days after enucleation of the eye. The myelin sheaths were relatively well preserved although some were distorted. Proliferation of fibrous astrocytes was apparent at 15 days and was increased at 45 days. Phagocytosis of degenerating myelin was not evident until 100 days and was pronounced by 200 days. Some axonal debris was still noted within an occasional residual myelin sheath at 100 days, a few of the sheaths having maintained a semblance of their usual form. Gliosis was striking in degenerating optic nerves at both 100 and 200 days. A difference in the rate of removal of myelin in degeneration of a peripheral nerve, tibial, in comparison with a central tract, optic nerve, was noted. In peripheral nerve, phagocytosis and breakdown of myelin were much more rapid, and little residual myelin debris remained at 100 days.

NUTRITIONAL DEFICIENCIES THAT IMPAIR AXONAL REGENERATION AND REMYELINIZATION AFTER WALLERIAN DEGENERATION IN RATS, WITH SPECIAL REFERENCE TO VITAMIN B₁₂ AND PYRIDOXINE. F. Stephen Vogel,* The New York Hospital-Cornell Medical Center, New York, N.Y.

Degenerations of myelin and axis cylinders, occurring together or separately, characterize certain nutritional deficient states in human beings. It is well established that, after Wallerian degeneration in crushed, non-severed nerves of well-nourished animals, the axons regenerate and myelin is laid down about them in orderly and predictable manner. To learn more about the nutritional requirements of myelin and axons, a study was made of the effects of specific nutritional deficiencies upon these regenerative processes.

Groups of male rats, weighing 60 gm., were fed diets lacking or markedly deficient in one of the following: thiamine, riboflavine, pantothenic acid, pyridoxine, vitamin B₁₂, folic acid, choline, protein, copper, or potassium. Serving as controls were groups of animals given completely fortified diets either ad libitum or in restricted amounts, causing weight losses comparable to those of the experimental groups. After 2 to 4 weeks, the upper segment of the right sciatic nerve of each animal was crushed. The extent of axonal regeneration and the degree of remyelination were determined 40 days later, by histologic means, using Cajal's silver nitrate method for axis cylinders and the Weil-Loyez, Spielmeyer, Masson, oil

red O, and osmic acid methods for myelin. The concentration and the maximum diameter of the axis cylinders and of the myelin sheaths were determined by a filomicrometer. Selected nerves were examined, in addition, by electron microscopy.

The regenerating nerves of animals deficient in vitamin B₁₂ regularly showed a conspicuous lack of remyelination without impairment of axis cylinder regeneration. The myelin sheaths were sparse and extremely slender, measuring less than 0.5 μ in width, and they rarely extended 15 mm. beyond the compression line. By contrast, the regenerating nerves of animals on the same diet supplemented by crystalline B₁₂, contained numerous myelin sheaths measuring up to 1.5 μ in width.

In animals depleted of pyridoxine, the regenerating nerves were regularly characterized by a paucity of axis cylinders and, associated with this, scant myelin formation. Micrometric counts disclosed approximately one fourth as many regenerating axic cylinders in the deficient as in the control animals.

These observations provide evidence that Vitamin B₁₂ plays an important rôle in the metabolism of myelin. This has particular interest in relation to the demyelination that characterizes the neurologic lesions in pernicious anemia. The results also link pyridoxine with the metabolism of the regenerating neuron, but fail to show any such relationship with thiamine, riboflavine, pantothenic acid, folic acid, choline, exogenous protein, copper, potassium, or with starvation.

A VASOTOXIC REACTION IN THE CANINE SPINAL CORD: IN VIVO OBSERVATIONS.† George Margolis,* Ashton T. Griffin, George T. Tindall, Patrick D. Kenan, and Roy Wood, Duke University School of Medicine and Hospital, Durham, N.C.

Roentgen contrast agents injected into the aorta in toxic concentrations produce a severe destructive effect upon the spinal cord of dogs. An understanding of the mechanism of this vasotoxic effect was approached by a study of the circulatory dynamics of the spinal cord, under normal conditions, and during and following the primary circulation of nontoxic and toxic concentrations of contrast media. Particular attention was focused upon the temporal aspects of the circulation, vascular permeability, vascular caliber, character of blood flow, and integrity of circulation following a toxic injection. A fluorescein, activated by ultraviolet light, served as a marker for injected agents and as a permeability indicator. A typical vasotoxic reaction is characterized by: (1) an immediate stasis of the cord circulation, the primary circuit of the injection mass being prolonged about five times the normal application period; (2) an immediate break in the permeability barriers of the pial vascular network; (3) an immediate onset of sludging of blood in the injured vascular bed; (4) an immediate functional shutdown of the regional arterial afferent radicles to the spinal cord. This shutdown may last several hours, but is unassociated with demonstrable thrombus formation. Meanwhile, the longitudinal vascular channels of the cord maintain an active surface circulation. No vasospastic effects upon the cord vessels have been visualized during this toxic reaction. The toxic reaction was unrelated to significant changes in systemic blood pressure.

This observational method allows a dynamic approach to the study of the pathologic physiology of the circulation of the central nervous system, under the influence of a broad variety of noxious stimuli.

A FAMILIAL FORM OF DIFFUSE SCLEROSIS ASSOCIATED WITH MARKED CEREBELLAR CHANGES. Joseph F. Smith,* University of Cincinnati College of Medicine, Cincinnati, Ohio.

Non-identical twins are described in which death occurred at 6 and 6½ months

† Supported by a research grant (B 1102) from the National Institutes of Health and by a research grant from the National Paraplegic Foundation.

after a similar illness in which twitchings and convulsions were followed by drowsiness, lethargy, and decerebration. The total duration of illness in each instance was almost 3 months, the first 3 months of life being apparently normal.

The changes in the brain in each were similar and included very deficient myelination throughout the cerebrum with scanty changes in some parts of the deeper gray matter. The latter comprised occasional cystic changes with astrocytic proliferation and preservation of neurons. A similar focal cystic change was seen in some of the central nuclear masses. In the white matter a fine porosity was evident, with very few myelin sheaths present, more numerous axis cylinders and astrocytic proliferation, and but little gliosis. The optic nerves, optic chiasm, and corpus callosum were involved in the process and the internal capsules to a lesser degree. The cerebrum was only slightly reduced in size and although the white matter was obviously abnormal to the naked eye, the gross appearance of the cerebellum was even more so. In both examples the folia of the cerebellar cortex appeared thin and shrunken and in one, distention of folia at the posterior borders of the hemispheres produced cysts up to 0.5 to 1 cm. in diameter.

Microscopy indicated a marked edema of the white matter extending into the cortex with great loss of granular layer cells but relatively good preservation of the Purkinje cells. Myelination had taken place to a greater extent than in the cerebrum but many sheaths were damaged. Astrocytic proliferation was prominent in both granular layer and white matter and in the latter was accompanied by numerous glial fibrils. Some evidence of myelin catabolism was present in the cerebrum but no lipid was demonstrated in neurons. Metachromatic material was absent from cells and surrounding tissue and the characteristic cellular reaction and globoid bodies of Krabbe's form of diffuse sclerosis were not present.

It is concluded that the present two examples are forms of diffuse sclerosis in which a familial history, often with a Jewish ancestry and consanguinity, are common. A marked failure of myelination throughout the white matter with cystic change, evidence of myelin catabolism in some, astrocytic proliferation but scanty fibril formation, and conspicuous changes in the granular layer of the cerebellum are the salient features. About 12 cases are reported under various titles in the world's literature.

HISTOGENETIC APPRAISAL OF GLIOBLASTOMA MULTIFORME. I. Costero,* National Institute of Cardiology, Mexico City, Mexico.

The behavior of glioblasts in tumors is very peculiar. In glioblastoma multiforme one always finds two varieties of neoplastic glioblasts: small, pale, bipolar spongioblasts and large, eosinophilic, piriform astroblasts. These two varieties of neoplastic glioblasts are permanently associated with one another through transition forms. The small, pale, bipolar spongioblasts produce acellular areas and necrotic zones in the tumor parenchyma, and stimulate characteristic vascular proliferation in the stroma. Large, eosinophilic, piriform astroblasts turn their main cytoplasmic prolongation toward capillary vessels forming "gliovascular systems" and tend to become multipolar astrocytes. Another important property of neoplastic glioblasts is their ability to show anaplasia, resulting in adendritic, spheroidal, eosinophilic astroblasts and in multipolar, monstrous, polychromatophilic astrocytes.

In a few special cases the glioblastoma may be formed predominantly by spongioblasts, astroblasts, or astrocytes. This cellular polymorphism of glioblastomas may relate to its degree of malignancy. In a tentative schematic form, one can state that anaplastic, spongioblastic, astroblastic, and astrocytic glioblastomas represent the order from major to minor degrees of malignancy. But in every case a minute analysis demonstrates the simultaneous presence of spongioblasts and astroblasts in glioblastomas. In other words, astrocytes in glioblastomas come from two morphologic varieties of glioblasts—spongioblasts and astroblasts—both equiv-

alent from the point of view of their oncogenetic properties and representing two non-separable progressive grades of maturity in which spongioblasts are the first and astroblasts the last.

ANOXIC NECROSIS OF LIVER IN MAN. I. N. Dubin,* Woman's Medical College of Philadelphia, Philadelphia, Pa.

Anoxic necrosis of liver is a common lesion in man and animals and generally goes unrecognized. It has been mistakenly attributed to chemical poisons, viruses, bacteria, viral and bacterial toxins, and, when massive, to arterial occlusion and even to a specific lesion of eclampsia.

The nature and distribution of the lesion is best explained by Rappaport's concept that the so-called liver lobule is made up of several circulatory sublobular units; each unit is triangular, with the base at the periphery of the lobule and the apex at the efferent central vein (tributary of the hepatic vein). The smallest lesion of anoxic necrosis is comprised of a roughly triangular area which abuts but does not encircle the central vein of the lobule, i.e., an area of paracentral necrosis at the terminus of one sublobular unit. If enough sublobular units are involved, the lesion becomes "centrolobular." If anoxia is severe, and particularly if collapse of circulation is extreme (as in prolonged shock), the entire lobule may undergo necrosis, and there may even be massive areas of liver involved by this necrotic process, simulating massive arterial occlusion.

This lesion may be seen in many forms of anoxia, such as high altitude flying, neonatal anoxia, and shock from exsanguination and other causes. Probably the hepatic lesion in most cases of eclampsia may be explained on this basis. A special form of this anoxic necrosis is the infarction of pseudolobules in portal cirrhosis. In this disease the pseudolobules are inadequately supplied with blood and are vulnerable to the slightest drop in blood pressure. In acute cardiac failure probably two factors operate in the production of centrolobular necrosis of liver, one being the centrolobular passive congestion and hemorrhage, the other being anoxic necrosis.

The necrosis may take at least two cytologic forms. (1) Vacuolization of cytoplasm, even to the point of marked ballooning of cells. The vacuoles do not contain fat or glycogen and probably consist of water-logged sacs. (2) Acidophilic shrinkage of cells, leading to coagulation necrosis. This is the more common form. The necrotic tissue is then invaded by polymorphonuclear leukocytes, a process which may be so extensive as to resemble microabscesses.

LIVER NECROSIS AND RETICULO-ENDOTHELIAL CELL CHANGES IN PREGNANT MICE RECEIVING CHLOROPHYLLIN. William Antopol* and Susi Glaubach, Beth Israel Hospital, New York, N.Y.

It has been reported previously that chlorophyllin, administered subcutaneously to normal mice, is taken up by all tissues except brain and lung. Forty-eight hours after the injection the organs are stained deep green, and the color is still present after 6 months. In the liver, the pigment lodges in the Kupffer cells. In the present study, pregnant Paris mice carrying mammary carcinoma agent were subcutaneously injected with chlorophyllin. Areas of necrosis in the liver, similar to those produced by chlorophyllin in C57 mice bearing carcinoma 755, were found. The mice received one injection of 20 mg. of chlorophyllin 5 to 8 days before parturition and were sacrificed after 1 to 6 months.

Gross examination of the liver revealed irregular, flat, depressed, deep green areas. On section the deep green areas involved the superficial 1 to 2 mm. of the organ, while the remainder of the liver tissue was pale green. Microscopic examination revealed extensive areas of necrosis, corresponding with the depressed deep green areas. Adherent thrombi in varying stages of organization were found in

blood vessels in and about the necrotic zones. Most of the viable liver cells were small, but occasional groups of large cells with pale, finely granular cytoplasm were present. An occasional mitotic figure was seen. The Kupffer cells were enlarged, rounded or polyhedral, and stained green; in places they formed nests of very large cells resembling the cell formations in Gaucher's disease. The reticulo-endothelial cells in the other viscera contained only an occasional large cell resembling those found in the liver. These findings contrast with those in normal mice in which necrosis is absent and the reticulo-endothelial cells are only slightly enlarged. Also noteworthy is that the offspring of the Paris mice treated with chlorophyllin during pregnancy were normal despite the liver changes and the deep green color of the mammary tissues.

STRUCTURE AND DISPOSITION OF HEMOSIDERIN IN CELLS AS DISCLOSED BY ELECTRON MICROSCOPY: RELATIONSHIPS OF FERRITIN AND HEMOSIDERIN. G. W. Richter,* Cornell University Medical College, New York, N.Y.

Typical isotropic, iron-positive, hemosiderin granules, present in cells of the kidney, liver, and spleen of rats given repeated intraperitoneal injections of rat hemoglobin, were found to contain innumerable, very electron-dense particles, and clusters of such particles, embedded in non-electron-dense matter. The discrete particles had a mean diameter of 55 Å, and a size-frequency distribution indicating uniformity, and corresponding with the size of the quadruplet of iron micelles found by Farrant in crystals of ferritin. Hemosiderin granules in hepatic and reticulo-endothelial cells of rats that had been given ethionine during a period of 2 months contained similar electron-dense particles, many with diameters between 50 and 60 Å. In both groups of rats such particles often were scattered diffusely through the cytoplasmic "matrix" of cells containing hemosiderin granules.

Biopsy specimens from the liver and spleen of a patient with advanced hemosiderosis showed iron-positive hemosiderin granules that contained similar electron-dense particles. Most of these had diameters of 30 Å or integral multiples of 30 Å, and presumably represented single and multiple iron micelles, respectively. Many particles with diameters of approximately 60 Å were scattered through the cytoplasmic matrix.

By means of cadmium sulfate, ferritin was crystallized from the livers, spleens, and kidneys of the hemosiderotic rats with ease, but could not be crystallized from comparable amounts of tissue from untreated control rats; and it was crystallized also from the spleen of the patient with hemosiderosis. The findings indicate that the electron-dense particles in hemosiderin granules are iron micelles and clusters of iron micelles, and that ferritin itself may be a component of hemosiderin granules.

In hemosiderin-laden cells of the rats given hemoglobin or ethionine, dense aggregates of the electron-dense particles often were present within discrete cytoplasmic bodies that were delimited by double membranes, and sometimes contained "cristae." Often the membranous bodies were disrupted and seemed to be discharging electron-dense particles into the surrounding cytoplasm. The term siderosomes is proposed for these specialized cytoplasmic structures, which may be derivatives of mitochondria, and apparently play a part in the formation of hemosiderin.

NECROPSY FINDINGS IN A WHITE MAN WITH THALASSEMIA MINOR—SICKLE CELL TRAIT WITH FATAL CRISIS PRECIPITATED BY AIRPLANE FLIGHT. Morgan Berthrong,* James H. Donald, Carlos Perez-Mesa, and Gustavo Ganem, Glockner-Penrose Hospital, Colorado Springs, Colo.

In recent years it has become apparent that while patients with minor defects

or traits of either Mediterranean anemia or sickle cell disease usually will show no manifestation of disease, combinations of the traits in the same patient will often result in a serious hematologic disorder. It also has been reported that patients who have sickle cell trait alone may suffer splenic infarcts due to a sickle cell crisis if exposed to high altitudes.

We wish to present the necropsy findings in a 42-year-old white patient of Greek ancestry who had a combination of thalassemia minor and sickle cell trait and who succumbed with diffuse vascular occlusions consequent to a sickle cell crisis which followed an airplane trip. He had had a long history of anemia considered to be a mild sickle cell disease. He had led a reasonably active life until his final acute illness. Examination of red blood cells just prior to death revealed marked sickling. Hemoglobin paper electrophoresis showed a combination of S and F hemoglobins.

Necropsy disclosed massive old splenic infarctions, recent and old small cerebral, renal, and myocardial infarctions, and multiple minor infarcts of other viscera. Cirrhosis of the liver of the type seen in sickle cell disease was present as well as the peculiar nephritis found in this condition.

The paper electrophoretic pattern of the hemoglobin was characteristic of the combination of thalassemia minor and sickle cell trait. The case would otherwise have been considered one of sickle cell disease with an abnormally long survival. The importance of the combination of minor defects is emphasized. The case also points out the possibility of a fatal sickle cell crisis following airplane travel by patients with various sickle cell diseases. Cases thus far described in the literature have been examples of splenic infarction alone.

THE HISTOPATHOLOGY OF CONGENITAL BILIARY ATRESIA. Daniel Stowens, Armed Forces Institute of Pathology, Washington, D.C.

Differentiation of jaundice in the newborn period represents a medical emergency. Among the conditions leading to prolonged progressive icterus, congenital malformations of the bile ducts are among the more serious. Diagnosis has rested, and to a large extent still does rest, upon actual visualization of the structures in the porta hepatis. However, such examination may be misleading and dangerous. This report concerns the histopathologic characteristics of congenital biliary atresia as determined by biopsy and necropsy of 75 cases. It has been determined that the malformation is divisible into six basic types, all of which manifest themselves differently in the liver. Only one of these is amenable to surgical correction. The clinical embryologic and physiologic implications of the disease are discussed.

STUDIES ON OCHRONOSIS. Thomas J. Moran,* Presbyterian Hospital, Pittsburgh, Pa.

A case of hereditary ochronosis in a 48-year-old woman with death from ochronotic nephrosis is presented. Histologic comparisons of the ochronotic pigment with melanin from normal skin and melanin from a pigmented nevus were carried out by special stains and electron microscopy. The ochronotic pigment was extracted and isolated from cartilage, liver, and kidney. Certain physical and chemical properties of the isolated ochronotic pigment are reported and compared with comparable data of melanin pigment from a malignant melanoma.

HUMAN TISSUE RESPONSE TO ALUMINUM POWDER APPLIED TO THE EDGES OF FISTULAS. Amour Fiscus Liber,* Bronx Veterans Administration Hospital, New York, N.Y.

Metallic aluminum powder is now widely used to protect the skin about the edges of draining wounds and fistulas. Specimens available for this study include

a ureterostomy, a jejuno-uretero-cutaneous fistula, a gastrostomy, and a jejunostomy. The first two were in the same patient. In all but the last, aluminum particles had penetrated to varying depths into both subcutaneous, adipose, and connective tissues. In the gastrostomy case, aluminum was found at least 4 mm. from the stoma in the wall of the stomach.

Response to the metal was characterized mainly by fibrosis, with occasional small areas of plasma cell infiltration. Only one particle had a foreign body giant cell adjacent to it, and this may have been elicited by suture material. That the patients were able to produce a granulomatous reaction was shown by the abundant giant cell reaction to suture material in all cases, and to fat necrosis in one. In the gastric tissue aluminum particles lay in the muscularis propria and were surrounded by patches of fibrosis replacing smooth muscle. There is no evidence that the powder interfered with healing or carried micro-organisms into the tissues.

Aluminum powder is made up of exceedingly thin, flat, sharp-edged plates. This probably accounts for their ability to penetrate into tissues. The particles have a characteristic microscopic appearance, and are readily recognized by their shape and by a luster manifested as the plane of focus is varied slightly.

ANAPHYLACTIC SHOCK AND ASCORBIC ACID. Adolf Hochwald* and Steven Frank, Ray Brook State Tuberculosis Hospital, Ray Brook, N.Y.

A number of years ago one of us reported the inhibition of anaphylactic shock, *in vivo*, by large doses of ascorbic acid injected shortly prior to the challenging antigen injection. The experiments were interpreted as indicating inhibition of release of H-substance after injection of ascorbic acid or other substances of high redox potential. Sensitization was not inhibited. The more recent interest in the effects of cortisone on the phenomena of hypersensitivity suggested a re-evaluation of the earlier experiments with ascorbic acid. As a preliminary step the validity of the earlier findings was tested by experiments *in vitro*, on the isolated guinea pig intestine.

Intestinal strips exposed to ascorbic acid *in vitro* showed an appreciably diminished response to antigen in the Dale bath, when compared to strips of the same animal not exposed to ascorbic acid. Intestinal strips tested with histamine in similar fashion gave inconsistent results; they exclude, however, a diminution of the muscle sensitivity as explanation of the findings with the testing of antigen.

THE STRUCTURE AND CHEMICAL COMPOSITION OF RESIDUAL LIPID PLAQUES IN RABBITS FOLLOWING PRANDIAL (CHOLESTEROL) HYPERLIPEMIA. Frederick C. Bauer, Jr.,* Richard Nailor, and Edwin F. Hirsch,* St. Luke's Hospital, Chicago, Ill.

Present concepts in the evolution of atherosclerosis relate the disorder to lipid factors and tissue factors.

The prandial (cholesterol) experimental atherosclerosis in rabbits is associated with a marked hyperlipemia in which the blood lipids have a disproportionately high content of cholesterol ester and free cholesterol. The quantitative amounts of the neutral fat, the phospholipid, and the cholesterol (free and esterified) fractions of the hyperlipemia are expressed in a solvent (neutral fat—phospholipid) to solute (cholesterol) ratio. Emulsified blood lipids with this composition, in the rabbit, have the physical properties of a particulate substance which stimulates phagocytosis (lipophages). Graphs and charts record the composition of the blood lipids through the hyperlipemic phase and until the lipids return to normal. Aortic plaque residues are chemically analyzed and tissues examined histologically for residual plaque deposits. Tissue reactions produced experimentally in the lungs of rabbits with lipid emboli of chemical compositions similar to those of the hyperlipemic blood lipids and in the plaques of the aorta are compared.

ENCrustATION AND PERMEATION OF BLOOD PROTEINS IN THE GENESIS OF ARTERIOSCLEROSIS. Robert H. More,* and M. Daria Haust, Queen's University Faculty of Medicine, Kingston, Ont.

In the pathogenesis of arteriosclerosis, lipid is, by most workers, believed to be selectively imbibed and deposited in the intima of the artery, other components of the blood being neglected. Our study in the past 2 years on various lesions of arteriosclerosis in the aorta and coronary arteries, led us to the conviction that there are other components of blood, namely the proteins, which play possibly an equal rôle in the inception and progress of the common arteriosclerotic lesions. Blood proteins were found either precipitated on the vessel surface (thrombi) or insuded into the vessel wall.

This protein material, in either form, was converted by means of organization into components of the vessel wall, through the stage of AMP-rich ground substance followed by development of collagen, elastic and reticulum fibers. The ageing of such an area resulted in a sclerotic white plaque, which often contained remnants of unorganized protein material.

While the superficial encrustation appeared to be more common in the aorta, the serous insudation was most common in the coronary arteries. The latter fact, viewed from the concept of Rossle's serous inflammation, and the fact that the process of arteriosclerosis is of an episodic, relapsing type, may provide reason to consider this disease within the scope of inflammation. It is our impression from these observations that the important intimal thickenings of aorta and coronary arteries are, in large measure, due to the conversion of blood proteins, present either on the surface or in the intima, into intercellular connective tissue.

RÔLE OF SALT IN THE PRODUCTION OF NECROTIZING VASCULAR DISEASE. Simon Koletsky,* Western Reserve University School of Medicine, Cleveland, Ohio.

Arterial and arteriolar necrosis was induced in the rat by means of renal injury. It was found that the development of vascular lesions was critically influenced by the amount of dietary salt available to the animals. With salt loading, the vascular change was considerably augmented in severity, whereas restriction of sodium chloride had the effect of reducing or eliminating the lesions.

Evidence was obtained also that the sodium chloride served to complement renal injury in the production of vascular disease. Mild damage to the kidney plus high salt intake yielded lesions which did not occur when the intake of salt was normal or low. On the other hand, severe kidney damage induced necrosis of vessels in spite of drastic salt restriction.

The significant rôle which sodium chloride appears to play in the pathogenesis of necrotizing vascular disease suggests that the vascular lesions develop through renal dysfunction in the handling of electrolytes which in turn leads to an imbalance of cellular cations in the vascular bed.

THE SPECIFIC LOCALIZATION OF ANTIGEN IN LESIONS OF EXPERIMENTAL SERUM SICKNESS. Charles G. Cochrane, Jacinto J. Vazquez, and Frank J. Dixon,* University of Pittsburgh School of Medicine, Pittsburgh, Pa.

The present study was undertaken to analyze hypersensitive lesions of the serum sickness type for specific antigen and antibody. Albino rabbits were given a single intravenous injection of purified bovine serum albumin (BSA), 250 mg. per kg., labeled with I^{131} . Animals were sacrificed between 4 days after injection and 7 days after complete elimination of I^{131} BSA from the circulation. A majority of the lesions developed shortly before the time of complete antigen elimination, while antigen-antibody complexes were present in the circulation. The lesions

produced included a proliferative glomerulitis, coronary arteritis, and an endocarditis.

Using the fluorescent antibody technique of Coons, it was possible to demonstrate concentrations of the BSA within diseased glomeruli, as well as concentrations of both antigen and the host's own gamma globulin in the lesions of the arteries. Attempts to identify specific antibody (anti-BSA) in these lesions have been unsuccessful to date. Concentrations of BSA were not observed in sites other than the active lesions. In rabbits injected with BSA and examined prior to the development of lesions, or in irradiated rabbits not developing serum sickness after injection of BSA, no histologic accumulations of BSA or the host's gamma globulin were seen. Further control animals, injected with an unrelated foreign protein (human gamma globulin) during the development of serum sickness resulting from a prior injection of BSA, showed accumulation of BSA but did not show human gamma globulin in the lesions. This indicates that the localization of antigen was specific and not a non-specific outpouring or trapping of protein in the lesions.

These studies demonstrate that antigen is specifically localized in the lesions of serum sickness at the time they are produced. The relationship of these findings to the pathogenesis of lesions of serum sickness is discussed.

WATER AND SODIUM CONCENTRATIONS IN THE AORTA OF THE RABBIT AND THEIR RELATION TO BLOOD PRESSURE.† James R. Stuart, Douglas Waugh,* and Arlene J. Maximchuk, McGill University Faculty of Medicine, Montreal, Que.

Bilateral silk wrapping of the kidneys of young male rabbits produced a marked degree of acute hypertension. Three weeks after the wrapping the mean systolic blood pressure was 34 per cent higher than that of the controls, and the difference was highly significant ($p < 0.001$). At that stage the animals were killed, and the water and electrolyte concentrations in the aortas and other tissues determined. In the aortas, the water and sodium concentrations were slightly, but not significantly, increased over the control levels, and the potassium concentration was unchanged.

By contrast, the aortas of untreated rabbits 3 to 5 years old showed a much greater and highly significant increase in sodium, and to a lesser extent, of water, with no change in potassium; however, the blood pressure of rabbits of this age is only slightly higher than that of young animals and considerably lower than that of the acutely hypertensive group referred to above.

The results suggest that the systolic blood pressure of the rabbit is not closely related to the total water or sodium concentration in the aorta.

AN INJECTION STUDY OF THE MESENTERIC ARTERIES.‡ Leopold Reiner,* Felix L. Rodriguez, and Rudolph Platt, Beth Israel Hospital and Harvard Medical School, Boston, Mass., and the Bronx Hospital and Albert Einstein College of Medicine, New York, N.Y.

The mesenteric circulation of 49 unselected and 5 selected adults was studied at necropsy by a technique of (a) injection of the celiac, superior mesenteric, and inferior mesenteric arteries; (b) partitioning of the viscera in accordance with the respective arterial arborizations; (c) x-ray photography, and (d) dissection of the arteries. Atherosclerotic plaques and narrowings were recorded for the three main stem arteries and for 20 of their determinate branches. The arteries most frequently involved were, in order, superior mesenteric, splenic, inferior mesenteric, celiac, gastroduodenal, and superior hemorrhoidal.

† Supported by a grant from the National Research Council of Canada.

‡ Supported by Grant H-2542 of the National Heart Institute, National Institutes of Health, United States Public Health Service.

Of 44 cases showing mesenteric arteriosclerosis, one third had only non-stenosing plaques whereas two thirds had between 1 and 76 points of narrowing distributed over as many as 16 arteries. Ten of the 44 cases had recent or old occlusions in the main stems, branches, or both, as follows: celiac, 3 times; superior mesenteric, 8 times; inferior mesenteric, 5 times. In 5 cases occlusions were present in more than one territory. The number of occluded arteries ranged from 2 to 9 with an average of 3.9 per patient. Often it could not be determined with confidence whether the occlusions were caused by thrombosis or embolization. Frequently associated with mesenteric occlusion were auricular fibrillation, cardiac mural thrombi, ischemic manifestations of the myocardium, and vascular impairment in the lower extremities. The spectrum of intestinal abnormality (5 cases) included pseudomembranous inflammation, giant ulcers (colon), and gangrene. On the other hand, there was evidence (4 cases) that occlusions of the superior or inferior mesenteric arborizations including the main stems could be entirely asymptomatic or merely produce transient, self-limiting, and undiagnosed abdominal episodes. Accordingly, such occlusions gave rise to little or no anatomical damage in the intestine. In one case extensive occlusive disease of the gastroduodenal and pancreatic arteries was associated with marked fibrosis of the pancreas. In another there was evidence that the mesenteric arteries had participated in bypassing an occluded lower abdominal aorta (Leriche's syndrome). Collaterals about sites of occlusions developed as the result of conspicuous enlargement of the normally delicate arteries of the mesenteric adipose tissues.

COMPARATIVE SEVERITY OF ATHEROSCLEROSIS IN THE AORTA, CORONARY ARTERIES, AND CEREBRAL ARTERIES. Ira Gore* and A. E. Hirst, Jr., Veterans Administration Hospital, West Roxbury, Mass., Harvard Medical School, Boston, Mass., and College of Medical Evangelists, Los Angeles, Calif.

Atherosclerosis is commonly considered to be a generalized process, yet clinical problems arising from it are usually local. Pathologists are well aware of differences in the distribution of intimal disease which account for the varied clinical manifestations. However, they have been handicapped in expressing such differences quantitatively by the limitations of conventional highly subjective grading procedures. Accordingly, it seemed advantageous to utilize an appraisal procedure, better adapted to quantitation. Briefly, the appraisal, based entirely upon gross inspection, considers the surface area of involvement, the types and proportions of intimal lesions, and expresses the disease as a five digit profile. By weighting, arithmetically, for the area of involvement, and logarithmically, for the types of lesion, an atherosclerotic index is derived which forms the basis for comparison.

In this fashion, 572 routine unselected necropsies were examined, including 347 males and 225 females. In all, the disease in the major coronary arteries was compared with that in the aorta. In 267, 153 males and 114 females, a similar comparison was made between the major cerebral arteries and the aorta. The atherosclerotic index rose progressively with age in the three areas but lagged behind in the coronaries and even more so in the cerebral arteries. After the development of an appreciable degree of intimal disease, represented by an index of ten or more in one of the areas, disparities of 100 per cent or more were present in the following proportions: coronary arteries vs. aorta, 42 per cent (146/347) in males and 32 per cent (71/225) in females; cerebral arteries vs. aorta, 61 per cent (93/153) in males and 52 per cent (59/114) in females. In general, both coronary and cerebral atherosclerosis were less advanced than the disease in the aorta. However, in 32 of the males and 7 of the females, in cases of disparity, coronary atherosclerosis exceeded the aortic disease by more than 100 per cent. The difference suggests the importance of the male sex hormone in coronary atherogenesis especially since analysis by decade shows two thirds of the

male group to have been younger than 61 years. There was not a similar sex difference in cerebral atherosclerosis. In the disparate group, cerebral exceeded aortic disease in 5 males and 3 females.

ATHEROSCLEROSIS IN CAPTIVE WILD MAMMALS AND BIRDS.† M. T. I. Cronin and H. L. Ratcliffe,* Zoological Society of Philadelphia and University of Pennsylvania, Schools of Medicine and Veterinary Medicine, Philadelphia, Pa.

Atherosclerosis has occurred in a wide variety of mammals and birds that have died in the Philadelphia Zoological Garden. As a rule the frequency and extent of this disease among animals of any order or family has been proportional to the average length of life in captivity (exhibition periods). However, exceptions to this trend have occurred with sufficient frequency to suggest that other factors also influence its development. Hence we have attempted to determine whether in addition to age, factors such as sex, diet, crowding, and concurrent disease may contribute to the development of atherosclerosis.

The records used in this study were collected over a period of 40 years, 1916 to 1956. During the first half of this period the traditional and often inadequate diets common to zoological gardens were in use. In the second half, however, controlled diets have been fed.

The results of this analysis show that if the material be considered as a whole, age is the only factor that seems to be directly related to the development of atherosclerosis. The disease has occurred with about equal frequency in males and females, and may not be related to crowding, total population, diets or to concurrent disease. When, however, its development in particular orders, families, and genera are considered, there is an apparent relationship to crowding, diet, and concurrent disease.

ENDOCARDIAL SCLEROSIS IN INFANCY DUE TO ABNORMAL STORAGE (GARGOYLISM). Lotte Strauss* and Rudolph Platt, Mt. Sinai Hospital and Lebanon Hospital, New York, N.Y.

It is known that inborn errors of metabolism may involve the heart, as in cardiac glycogen storage disease and in gargoylism. While death from cardiac failure in early infancy is common in the former condition, it is considered very rare in gargoylism. The case of a 5-months-old girl with cardiomegaly, who died in congestive heart failure after a short period of illness, represents the second known example of diffuse endocardial sclerosis in infancy due to abnormal storage of the type seen in gargoylism. The child exhibited none of the striking features usually associated with this disease, and except for an upper respiratory infection, 3 to 4 weeks before death, she had appeared normal in every respect.

The necropsy, which excluded examination of the brain and eyes, revealed a markedly enlarged heart with diffuse endocardial fibrosis and slight thickening of the valves, as well as minimal "atherosclerotic" changes in the abdominal aorta. Gross findings also included enlargement of the liver, slight enlargement of the spleen, and bronchitis. Microscopic examination revealed structural alterations characteristic of gargoylism, especially in the endocardium, large blood vessels, liver, spleen, and periosteum. These consisted of a conspicuous accumulation of large vacuolated cells. In tissues of mesenchymal derivation these cells tended to be associated with an increase of fibers. There was fibro-elastosis of the parietal endocardium indistinguishable from other forms of endocardial sclerosis were it not for the vacuolated cells.

Because of the delayed recognition of the nature of this case, tissues were not preserved with a view to chemical or histochemical investigation. Most of

† Supported in part by United States Public Health Service Grant H-1979.

the intracellular material was dissolved by 10 per cent formalin and absolute alcohol. Recent investigations of the chemical nature of gargoylism have suggested storage of one or more water-soluble complex polysaccharides. This would be in accordance with the striking involvement of mesenchymal tissues, such as the cardiovascular and skeletal systems.

This case is presented as an incomplete form or forme fruste of gargoylism with early and severe cardiovascular involvement which was responsible for death. It is suggested that gargoylism be considered in cases of acyanotic heart disease with cardiomegaly in infancy.

INFLUENCE OF ANOXIA AND MUSCULAR CONTRACTION UPON GLYCOGEN OF THE MYOCARDIUM OF THE RAT.† Benjamin Wittels, Leopold Reiner,* Howard A. Frank, and George W. Curtis, Beth Israel Hospital and Harvard Medical School, Boston, Mass., and the Bronx Hospital and Albert Einstein College of Medicine, New York, N.Y.

The depletion of glycogen from the myocardium of the rat was studied with the McManus PAS and Best's carmine stains in tissue sections obtained under conditions of (a) anoxia and muscular arrest (postmortem autolysis), (b) systemic anoxia and continued muscular contraction (open pneumothorax), and (c) localized anoxia produced by acute ligation of the left coronary artery. Specimens taken during life were obtained by abrupt transventricular ablation which stopped ventricular contraction instantaneously.

Control specimens taken by this method showed no reduction of myocardial glycogen. Postmortem glycogenolysis began beneath the natural surfaces and required more than 90 minutes for completion. Systemic anoxia in conjunction with continued muscular contraction exhausted myocardial glycogen within 5 minutes, the depletion being either uniform throughout or accelerated in the mid-myocardium of the ventricles. Acute ligation of the left coronary artery produced deglycogenization within the nutrient territory of the ligated artery, demonstrable at 2 minutes and complete at 15 minutes. This deglycogenization was speediest in the periphery but spared a narrow subendocardial zone not spared in postmortem autolysis or systemic anoxia. The rate and topographic distribution of glycogenolysis following acute coronary arterial occlusion was thus intermediate between postmortem autolysis and anoxic heart action, and suggested that the ischemic heart muscle quickly lost its contractility. About 30 minutes after coronary arterial ligation the myofibers of the infarct territory again became PAS positive. This could be shown not to be due to glycogen. Disappearance of glycogen and the succeeding color changes might possibly serve as the histochemical indicators of ischemic injury following ligation of the coronary artery. The data here presented also suggest that the contradictory reports of the glycogen contents in human hearts may be explained in part by differences in degree of anoxia before cessation of heart beat.

MYOCARDITIS OF EXPERIMENTAL HYPERSENSITIVITY AND ITS RELATIONSHIP TO RHEUMATIC CARDITIS. Bernard M. Wagner,* K. C. Pani, and Peter W. Vanace, Children's Hospital of Philadelphia, Philadelphia, Pa.

Myocarditis has been produced in four series of rabbits by the intravenous administration of meningococcal toxin, horse serum, and lipopolysaccharide extracted from two different strains of *Escherichia coli*. An additional group of animals received 400 r. of whole-body irradiation and subsequently were inoculated with the various agents. The tissues were subjected to a variety of histochemical

† Supported by Grant H-2542 of the National Heart Institute, National Institutes of Health, United States Public Health Service.

procedures and compared with similar studies of human auricular appendages. A total of 512 appendages have been reviewed of which 130 have been analyzed by histochemical methods. In this latter group, 26 revealed the presence of Aschoff bodies and of this group 6 showed the classical findings of acute rheumatic carditis. The auricular appendages demonstrating Aschoff bodies were studied also by immunohistologic techniques utilizing fluorescein-labeled anti-human gamma globulin, C-reactive protein, streptolysin O, and streptococcal protein. The relationship of experimental myocarditis to rheumatic carditis will be discussed. It appears that the Aschoff body is a unique phenomenon and is not reproduced in its entirety by the experimental methods employed. Evidence will be presented indicating that rheumatic fever is a true "collagen disease."

OBSERVATIONS ON THE HISTOGENESIS OF RHEUMATIC LESIONS OF THE HEART.
Hemprova Ghosh, Washington University, School of Medicine, St. Louis, Mo.

In the course of the study of microscopic sections of the hearts of patients who died with a clinical diagnosis of acute rheumatic heart disease, evidence suggests that the pathologic changes were due mainly to degenerative and reactive phenomena on the part of the cardiac muscle cells.

When the degeneration of the cardiac muscle is more gradual, the well known Aschoff cells, so-called Anitschkow myocytes, and other less well known cells are demonstrated in transition, while still preserving the characteristics of their precursors, the myocardial cells. After detachment from their mother cells, they often show the adherent fibrillary sarcoplasm with or without demonstrable cross striation. So-called fibrinoid material in rheumatic lesions is believed to be fragmented and degenerated sarcoplasm, as in some areas one may see cross striations in them and their continuation with the preserved myocardial sarcoplasm.

The so-called Anitschkow myocytes, after they are free from their mother cells, show great power of mobilization and multiplication, mitotically and amitotically. A variety of cellular components other than Aschoff cells may arise from these myocytes, some mimicking polymorphonuclear leukocytes and other inflammatory cells. The affinity for special stains shown by this so-called fibrinoid material and other cellular components of Aschoff bodies shows similarity to that of the cardiac muscle cells. The rheumatic lesion with formation of Aschoff bodies in cardiac valves also is believed to arise in the same way from cardiac muscle cells in the proximal part of the valves. Cells apparently resembling connective tissue cells in the distal portion of the cardiac valves, which are said to be derived from cardiac muscle cells in embryonic life, may have potentiality to react in a similar way.

In an area of severe myocardial damage the prominent finding is partial to apparently complete destruction of myocardium with formation of wide spaces between the surviving muscle bundles. Loss of continuity of heart muscle may explain blockage in conduction system giving rise to different arrhythmias. Cells with tiny and deeply stained nuclei and with fibrillary and irregular cytoplasm are first to appear in those spaces. Sometimes these cells attach themselves loosely to one another, forming parallel lines in a spindled pattern. They often form closed channels, some of which show blood in their lumina. Attempted regeneration of cardiac muscle cells is seen in the newly formed cells. All the above mentioned changes in areas of severely damaged myocardium, though found in acute rheumatic heart disease, are not necessarily specific for this disease alone as they are observed in severe heart damage by other causes. In cases of rheumatic fever usually there is further development of these cells described above into so-called Anitschkow myocytes, Aschoff cells, and a variety of other cell structures with attempted formation of sarcoplasm.

In the evolution of this disease various phases of development and regression

of the lesion are noted on single slides in acute cases. Regression of the rheumatic lesion by gradual scar tissue formation is probably the usual end result, but evidence is presented that some cardiac muscle fibers regenerate.

THE MICROSCOPIC STRUCTURE OF SENILE KERATOSES AND BOWEN'S DISEASE AND THEIR RELATIONSHIP TO EPIDERMOID CARCINOMA OF THE SKIN. Wallace H. Clark, Jr.,* Julius Levy, and Clayton Overton, Tulane University School of Medicine, Charity Hospital of New Orleans, and Ochsner Foundation Hospital, New Orleans, La.

Senile keratoses are considered premalignant lesions and Bowen's disease is usually classified as a carcinoma-*in-situ*, which some believe progresses invariably to an invasive neoplasm. Our studies of these disorders indicate that senile keratoses present two rather distinctive microscopic patterns. In addition, it has been shown that there is a similar distribution of senile keratoses and invasive carcinomas over the face, neck, ears, and hands, while Bowen's disease is most commonly located on the trunk.

One variety of senile keratosis occurred almost exclusively on the dorsum of the hand. At the edge of these lesions, which arose from a thickened epidermis, a sharp line of demarcation separated the "normal" epidermis from the atypical. The entire thickness of the epidermis, including the stratum corneum, was changed. The cells of the basal layer and stratum spinosum showed loss of polarity, variation in nuclear size and shape, and increased cytoplasmic eosinophilia. The stratum granulosum was absent or poorly developed, while the stratum corneum was pale, contrasting sharply with the normally basophilic keratin in this area. A striking feature was the failure of the cells surrounding a sweat duct, passing through the epidermis, to be involved by the atypical changes. This contrasted with the hair follicle epithelium, which was frequently involved by the atypical changes. Senile keratoses occurring elsewhere usually arose from an atrophic epidermis. The initial change appeared to be primarily in the basal layer where loss of cellular polarity and nuclear changes were prominent. A small suprabasal vesicle occasionally formed. Later, lesions might show small finger-like epidermal projections into the corium and there overlay parakeratosis. Our impressions of the histologic features of Bowen's disease do not differ essentially from those provided by others.

The epidermis adjacent to invasive epidermoid carcinoma frequently showed the changes seen in senile keratoses, but in only one instance were there changes suggestive of a relationship to pre-existing Bowen's disease.

THE EFFECTS OF CORTICOTROPIN AND ORCHIECTOMY ON THE ADRENAL GLAND OF THE DOG: A CORRELATION WITH EXCRETION OF 17-HYDROXYCORTICOIDS, 17-KETOSTEROIDS, AND ADRENAL ZONE MEASUREMENTS. George J. Race,* William M. Nickey, Phillip S. Wolf, and Elmer J. Jordan, University of Texas, Southwestern Medical School, Dallas, Texas.

The zona fasciculata and reticularis have been considered respectively as possible sources for 17-hydroxycorticoids and 17-ketosteroids. This assumption is based on the hyperplasia of the zones which follows corticotropin therapy and atrophy, which follows hypophysectomy or cortisone. The reticularis has specifically been considered as a possible source of 17-ketosteroids since it has been found to be enlarged in cases of hirsutism and adrenogenital syndrome. To study these possible relationships in the dog, six animals were given corticotropin 40 units per day for 28 days. The excretion of 17-hydroxycorticoids and 17-ketosteroids was measured simultaneously. After sacrifice, all adrenal glands were weighed and cortical zones were measured. In an additional group, six male dogs were orchietomized and the excretion of 17-ketosteroids was followed over a 53 day period. In the

corticotropin treated dogs, the combined adrenal weights were significantly increased. The 17-hydroxycorticoids and 17-ketosteroids excreted were increased initially after the corticotropin but by the fourth week the level of both hormones was diminished. Microscopically, the adrenal glands showed statistically significant hyperplasia of the zona fasciculata with a lack of clear-cut separation of the zones and with depletion of fat from the cell cytoplasm. The orchiectomized dogs showed no significant changes in adrenal weights. The mean excretion of 17-ketosteroids fell immediately following orchiectomy, but later during the seventh and eighth weeks showed a slight rise; however, not to the pre-orchiectomy level. The zona reticularis after orchiectomy showed a slight mean increase in thickness not statistically significant. These correlative functional and histologic studies are considered to be circumstantial evidence possibly favoring the theory of functional zonation. It would appear to be highly advantageous to develop the microchemical techniques for the identification of aldosterone, 17-hydroxycorticoids, and 17-ketosteroids, *in situ*, within the separate zones of the adrenal glands using fresh adrenal slices, in order to prove or disprove the theory of functional zonation.

SEPARATION OF PARTICULATE CELL COMPONENTS IN THE ADENOHYPOPHYSIS: CERTAIN RELATIONS OF STRUCTURE TO FUNCTION. Donald D. Mark, Baltimore City Hospitals, Baltimore, Md.

Homogenates of the anterior pituitary gland have been differentially centrifuged to yield three separate cell fractions: nuclei, cytoplasmic granules, and supernate corresponding to "cell sap." Light and electron microscopic studies demonstrate the morphologic integrity of these fractions when compared with known cell structure.

Bioassays of the separate fractions indicate that gonadotrophic, thyrotrophic, and adrenotrophic activity is largely concentrated in the cytoplasmic granules. Growth activity appears to be dispersed in all fractions. Enzymatic studies show that the granules contain the most respiratory activity, and in this way at least are comparable to the mitochondria of the liver. Although analyses of particulate size in the granule fraction disclose a separation into three broad classes correlating with known cell types, data obtained by density gradient ultracentrifugation reveals that the particles have a similar specific gravity.

STUDIES ON THE PATHOGENESIS OF "HYDROPIIC DEGENERATION" OF THE PANCREATIC B CELLS. Sydney S. Lazarus and Bruno W. Volk,* Jewish Chronic Disease Hospital and Albert Einstein College of Medicine, New York, N.Y.

Hydropic degeneration or vacuolization of the pancreatic B cells as observed in diabetes has been considered generally to be a result of functional exhaustion of these cells and a reversible state which may, if prolonged, lead to their destruction. "Functional exhaustion" does not, however, explain the vacuolization of pancreatic ductular epithelium which is also frequently observed in diabetes. Furthermore, hydropic degeneration has been shown to consist actually of deposits of glycogen within the cells. More recently it was observed that in cortisone-induced diabetes in rabbits, ductular glycogen infiltration precedes that in B cells. These findings seemed to negate the "functional exhaustion" theory of the pathogenesis of hydropic degeneration.

In the present study the sequential development of glycogen infiltration in the dog pancreas during experimental diabetes was studied. For this purpose two groups of normal adult mongrel dogs were used. The first group received 3 mg. per kg. of body weight of purified pituitary growth hormone subcutaneously for periods of up to 14 days. The second group was subjected to 85 per cent partial pancreatectomy. After a recovery period of 1 week the animals received 8 mg.

per kg. of body weight of cortisone acetate intramuscularly for periods of up to 4 weeks. The dogs were sacrificed at varying intervals and the pancreatic structure compared with the degree of diabetes.

It was found that some glycogen is present normally in pancreatic ductular epithelium but not in B cells. During the development of diabetes there is an increase in the vacuolization (glycogen infiltration) of the ductular epithelium prior to and generally in the absence of the appearance of glycogen in the B cells of the islets. Glycogen was found in the B cells only as a comparatively late phenomenon.

These findings, which are similar to those in the rabbit, make it unlikely that hydropic degeneration of the B cells is due to functional exhaustion. Furthermore, it negates the concept that this histologic lesion is a degenerative process. It seems more logical to consider it as merely one facet of the widespread increased glycogen deposition which occurs in diabetes similar to that in the renal tubular epithelium and the heart. The question is raised as to whether the glycogen infiltration is really part of the pathogenetic mechanism which leads to ultimate destruction of the B cells and to permanent experimental diabetes.

THE SO-CALLED CHRONIC PASSIVE CONGESTION OF THE PANCREAS. J. F. A. McManus,* University of Alabama Medical Center, Birmingham, Ala.

In 1925, von Glahn and Chobot described the histologic alterations in the pancreas in chronic passive congestion as atrophy of some of the acinar cells away from the islets with preservation of the acini in the area around the islets. This appearance was described independently by MacCallum and similarly blamed on chronic passive congestion. While it is unlikely that the change in the pancreas is related to chronic passive congestion alone, the association of the lesion with heart failure suggests that the pancreas may be useful as an indicator of the fashions in which heart failure produces signs and symptoms. This paper describes histologic and histochemical findings in the pancreas in heart failure, cirrhosis of the liver, and in Addison's disease, these three conditions representing various possible mechanisms for the change described as chronic passive congestion in the pancreas.

SO-CALLED THYROIDITIS: A CLINICOPATHOLOGIC STUDY AND CLASSIFICATION. Robert C. Horn, Jr.* and Joseph C. Sieracki, Henry Ford Hospital, Detroit, Mich.

All cases classified under "thyroiditis" or related diagnostic terms at the Henry Ford Hospital in the 4 years 1953 through 1956 have been reviewed with regard to clinical as well as pathologic aspects. An attempt has been made to classify the cases according to the scheme presented to the American Goiter Association by its committee of pathologists in 1956.

Granulomatous thyroiditis is not rare but other true inflammatory lesions (invasive fibrous thyroiditis and inflammations due to specific infectious or physical agents) are very uncommon.

The majority of cases comprising this study have been classified as lympho-epithelial goiter, including Hashimoto's struma. Deviations from the typical pathologic picture were numerous and there were many cases that could be fitted into the classification only with great difficulty or not at all. It was striking that certain instances of involuting hyperplasia were histopathologically indistinguishable from the characteristic lympho-epithelial goiter. This was especially true in cases in which anti-thyroid drugs were administered over long periods.

Although there were many instances in which a particular pathologic process was associated with a distinctive clinical picture, there were also many cases in which no such correlation was apparent.

AN ELECTRON MICROSCOPIC STUDY OF THE AGE CHANGES IN THE PROSTATE OF THE RAT AND A COMPARISON WITH PROSTATIC HYPERPLASIA IN MAN.† James C. Harkin, Washington University, School of Medicine, St. Louis, Mo.

In previous studies from this laboratory the structure of the prostate of the rat has been examined with the electron microscope. The internal structure is one of a complex group of ergastoplasmic sacs in addition to the nucleus, mitochondria, and a prominent supranuclear group of microvesicles, the Golgi complex. The general internal architecture of epithelial cells of normal prostate of the adult rat and human prostate in cases of hyperplasia are similar. Among the differences are a more prominent basement membrane and numerous electron-dense bodies, slightly larger than mitochondria, in the Golgi region of the human prostatic epithelium. Since prostatic tissues from man over a wide age range is not readily available for examination by electron microscopy, it seemed profitable to examine the prostates of rats of different ages and after castration.

The castrate prostate has epithelial cells with drastically collapsed sacs, reduced total mass of the cell, and electron-dense bodies. These electron-dense bodies have been found to correspond to accumulations of lipochrome pigment. They differ in location from those found in the cells of human prostate in that those in rat occurred between cells and in regions other than that occupied by the Golgi complex. In studying a series of rats of different ages it was found that with increased age there was a gradual deposition of opaque material in the region of the Golgi complex. Morphologically, these structures closely simulate the dense bodies in the prostate of man. The internal structure of the prostatic cells of rats of older ages continued to maintain the structure that we have come to associate with active secretion. These electron microscopic studies would seem to confirm further the impression that, with age, involutional changes are occurring in the prostate but that these changes do not appear to include loss of the cellular function of secretion.

HISTOLOGIC AND HISTOCHEMICAL OBSERVATIONS OF TISSUE CULTURE CELL LINES *IN VIVO*. Lewis L. Coriell, Robert M. McAllister, and Bernard M. Wagner,* South Jersey Medical Research Foundation, Camden, N.J., and Children's Hospital of Philadelphia, Philadelphia, Pa.

Modern tissue culture methods permit the serial cultivation of both cancer and normal cell lines in liquid media in large volume and presumably for indefinite periods. There appear to be no criteria which will definitely identify a cancer cell in tissue culture. The well established criteria used by pathologists in the diagnosis and prognosis of human malignant neoplasms are of little value to the tissue culture cytologist. In an attempt to determine the stability of cells maintained *in vitro*, a biologic method of assay was adopted. The following cell lines were studied: normal human conjunctiva (Chang), normal human kidney (Chang), normal human intestine (Henle), epidermoid carcinoma of the cervix, HeLa (Gey), and normal kidney of the monkey. All observations were made in parallel. These cell lines were inoculated into the anterior chamber of the eye and brain of the guinea pig; subcutaneously into monkeys, rabbits, chicks, and rats. Pretreatment of albino rats with 300 r. of total body irradiation and cortisone 2.5 mg. intramuscularly every other day for five doses, allowed for the reproducible development of subcutaneous tumors from all lines. The natural history of these tumors, including detailed histologic observations, will be presented. A variety of histochemical and enzymatic studies were applied. The relationship of these studies to cancer diagnosis and cell growth will be discussed. The extrapolation of the observations within

† Supported in part by United States Public Health Service Grant C-2836 from the National Institutes of Health.

the framework of human malignant disease emphasizes the frequent disparity between histologic and biologic malignancy.

MORPHOLOGIC AND BIOLOGIC PROGRESSION OF A LYMPHOID NEOPLASM OF THE MOUSE IN VIVO AND IN VITRO. Clyde J. Dawe* and Michael Potter, National Cancer Institute, Bethesda, Md.

Concomitant with progressive loss of lymphocytoid structure during repeated mouse passage, a methylcholanthrene-induced lymphoid neoplasm (P388) acquired increased ability to survive *in vitro*, where it changed morphologically to a reticulum cell sarcoma capable of continuous cultivation.

Originating in a DBA/2 mouse as a neoplasm clearly lymphocytic in structure, P388 was converted to ascitic form in the first mouse transfer. At the sixth mouse passage, alternate passage of cells through tissue culture and mouse was started, continuing through 17 cycles. During these culture attempts, the lymphoid character of the neoplasm was preserved with only slight variation, as observed both *in vivo* and *in vitro*. Maximum *in vitro* survival time in these passages was 17 days.

A second set of culture trials was carried out with cells from the 47th and later consecutive mouse passages. Here it was found that (1) the structure of P388 cells *in vivo* had altered toward a cell type corresponding to an atypical hemocytoblast, and (2) the *in vitro* survival time of these cells had increased to upwards of 30 days.

P388 cells taken from the 49th consecutive mouse passage were maintained in one culture for 51 days. During this period, neoplastic cells underwent morphologic alteration to a larger, more ameboid form containing many cytoplasmic vacuoles. The ascitic cells emerging from intraperitoneal inoculation of this culture into mice showed a correspondingly altered structure. They were much larger and more vacuolated, and many multinucleated forms were present. Cells isolated directly on glass from the ascites of this animal have been cultivated continuously for 5 months, through four serial subcultures. *In vitro*, the "derived" line (P388D₁) shows much pleomorphism with many spindle, stellate, multilobular, and multinucleated forms. These cells actively phagocytize carbon particles and in general behave as reticulum cells.

Continuous mouse passage of P388 was maintained in (BALB/c × DBA/2)F₁ hybrid mice. P388D₁ was found capable of killing DBA/2, (BALB/c × DBA/2)F₁, and (C57BL × DBA/2)F₁ mice, but not other strains lacking DBA/2 parentage, such as C57BL and BALB/c. This indicates that P388D₁ was derived from neoplastic cells of the original line of DBA/2 origin. 8.40×10^5 cells of P388D₁ killed (BALB/c × DBA/2)F₁ hybrids in 20.6 days (average), whereas, 1.35×10^5 cells of P388 at the 47th consecutive mouse passage killed similar hybrids in 11.8 days (average).

The *in vitro* conversion of P388 into a reticulum cell sarcoma has been repeated twice, the "derived" lines in the three instances showing characteristics differing only slightly from one another, and all three being thus far continuously cultivated.

HISTOCHEMICAL STUDY OF THE DISTRIBUTION OF ENZYMIC ACTIVITY IN MALIGNANT LYMPHOMA. Herbert Braunstein,* David G. Freiman,* and Edward A. Gall, University of Cincinnati College of Medicine, Cincinnati, Ohio.

Lymph nodes were procured shortly after surgical excision and prepared by freeze-drying and by appropriate fixation for histochemical demonstration of enzymatic activity. They were then studied for distribution of activity of non-specific esterase, alkaline phosphatase, acid phosphatase, phosphamidase, 5-nucleotidase, and, in a few cases, succinic dehydrogenase.

In normal and hyperplastic lymph nodes, histiocytes, both individually and

lining sinusoids, always demonstrated strong esterase and phosphatase activity and usually phosphamidase activity. Epithelioid cells and Langhans' giant cells behaved similarly. Alkaline phosphatase activity was confined to capillary (non-sinusoidal) endothelium and 5-nucleotidase activity to germinal centers of follicles. A preliminary report on findings in several different types of malignant lymphoma is presented. In general the enzymatic activity of cells of the various histologic types of malignant lymphoma corresponded to that of their normal counterparts. Of particular interest was the demonstration that the cells of reticulum cell sarcoma (histiocytic lymphoma) manifested enzyme activity identical with that of non-neoplastic histiocytes. Large anaplastic cells and Sternberg cells of Hodgkin's "granuloma" and sarcoma also manifested a similar pattern histochemically.

HISTOCHEMICAL DISTRIBUTION OF BETA-D-GLUCURONIDASE AND ALKALINE PHOSPHATASE IN MALIGNANT NEOPLASMS. Benito Monis and Alexander M. Rutenburg, Beth Israel Hospital and Harvard Medical School, Boston, Mass.

Beta-glucuronidase was assayed histochemically in 100 neoplasms using a modified post-incubation azo dye method. Enzymatic activity was quantitated according to color intensity and period of incubation. Seventy-eight of 83 tumors of epithelial origin stained intensely for beta-D-glucuronidase. Enzymatic activity was restricted to the cytoplasm of the tumor cells. Nuclei did not stain. All 17 mesodermal tumors (sarcomas) were largely negative for beta-D-glucuronidase. Normal epithelial cells were found to contain larger amounts of beta-D-glucuronidase activity than the mesodermal cells, with the exception of neutrophils which stained intensely.

In addition, 37 of the above neoplasms were assayed histochemically for alkaline phosphatase by a new simultaneous coupling azo dye method using naphthol AS phosphate as substrate. Tissue sections were incubated with this substrate and a suitable diazonium salt at pH 9.1, optimum for alkaline phosphatase. The naphthol AS liberated by enzymatic cleavage was sufficiently insoluble even at an alkaline pH and bound to tissue protein at or near the sites of enzymatic activity to preclude diffusion. It coupled promptly with the diazonium salt present in excess to yield a blue azo dye. Precise localization of enzymatic activity was achieved by deposition of this intensely blue precipitate at the sites of activity. Parallel sections were stained with Del Río Hortega's silver method.

The stroma in all 33 carcinomas stained intensely for alkaline phosphatase. Fibroblasts, collagen, capillaries, and small arterioles were very reactive. Tumor cells were negative. The scanty stroma and the tumor cells of two sarcomas (fibromyxosarcoma, sarcoma of the kidney) and two lymphocytic lymphomas were negative and only capillaries were stained. In addition, three other lymphomas stained for alkaline phosphatase by an azo dye method using 6-benzoyl-2-naphthyl phosphate as substrate showed the same distribution of enzymatic activity.

CYTOCHEMICAL STUDIES OF THE DEVELOPMENT OF THE VIRAL PAPILLOMA OF HUMAN SKIN. Gabriel C. Godman* and David P. Bloch, Columbia University College of Physicians and Surgeons, New York, N.Y.

Pathologic changes in the nuclei of infected cells of the verrucae which yield viral particles occur in a definite sequence of stages which can be followed progressively from the lower stratum spinosum to the stratum corneum. In the earliest stage, A, an acidophilic Feulgen-negative inclusion body is recognizable, and is distinct from the nucleoli. The inclusion body and nucleus subsequently enlarge; disorganization of chromatin structure and then margination become manifest (stages B and C). At stage D the eosinophilic inclusion bodies are about one third to one half the nuclear diameter, and the chromatin is entirely margined.

The inclusion grows and becomes basophilic and Feulgen-positive at stage E, and with the disappearance of the nucleus it comes to lie naked within the cytoplasmic remnant (stage F).

The relative amounts of Feulgen-colored DNA per cell at each stage in the development of the cellular lesion, as well as in normal appearing and hypertrophic (X) cells of the papilloma were measured microphotometrically. Determinations were made at a single wavelength through a "plug" or cylinder of the nucleus, and of whole nuclei with the "two wavelength" method to minimize distributional error. These were compared with measurements of the relative amounts of DNA in cells of the basal and spinous layers of normal human skin.

For cells of normal skin as well as the normal appearing cells in the papilloma, the frequency distribution curves, plotting relative amounts of DNA against number of cells, show the bimodal diploid and tetraploid peaks characteristic of growing tissues. With the advent of infection there is prompt synthesis of DNA in the nucleus, and levels from tetraploid to 16-ploid are already found in stage A, which is the earliest morphologically recognizable cytopathologic appearance. These amounts of DNA do not increase appreciably during the subsequent evolution of the cellular lesion. At a relatively late stage (E) all of the DNA becomes relocated ("transferred" or "reassembled" in the homogeneous inclusion body, whose range of relative DNA content at stage F is not significantly different from that of the preceding stages. Active neoformation of DNA appears to occur only in relatively intact nuclei. The inclusion bodies of infected cells were found to stain with the alkaline fast green technique for basic protein, and to contain protein in high concentration.

CERTAIN EFFECTS OF COLCHICINE, URETHANE, AND NITROGEN MUSTARD ON SELF-RENEWING TISSUES AND ON EXPERIMENTAL TUMORS.† Nathan B. Friedman* and Eileen Drutz, Cedars of Lebanon Hospital and University of Southern California School of Medicine, Los Angeles, Calif.

Nitrogen mustard interferes with the division of reserve cells in such self-renewing tissues as the seminiferous epithelium and the intestinal mucosa but does not inhibit the continued differentiation of postmitotic elements. Both colchicine and urethane interfere with differentiation. In experimental tumors colchicine inhibits cell division but permits the formation of polyploid nuclear structures. Nitrogen mustard inhibits the formation of nuclear material but permits development of the cytoplasm while urethane restricts both nuclear and cytoplasmic growth. It may be that certain agents interfere more with DNA than with RNA synthesis, that others primarily affect RNA metabolism, and still others inhibit both.

HISTOLOGIC EFFECTS OF THIOTEPA ON MALIGNANT TUMORS. Oscar B. Hunter, Jr.* and C. Barrie Cook, Washington, D.C.

ThioTEPA (triethylene thiophosphoramide), one of the newer mustard derivatives, has shown an increasing ability to inhibit the growth of a more widespread group of malignant tumors and to produce necrosis in these tumors in a selective fashion.

Study of lymphomas, carcinomas of the ovary, lung, stomach, liver, mouth, pancreas, and also melanomas, has shown appreciable inhibition of growth and necrosis of the tumor cells.

Judging from the histologic effects, the drug offers considerable promise as an adjunct to cancer therapy.

† Supported by United States Public Health Service Grant C-2490.

SPLENECTOMY IN MALIGNANT DISEASES OF THE RETICULO-ENDOTHELIAL SYSTEM. Bong Hak Hyun, James Butcher, and R. Philip Custer,* Presbyterian Hospital and University of Pennsylvania Graduate School of Medicine, Philadelphia, Pa.

Certain complications arise during the course of lymphomas, chronic leukemias, and erythremia that are incompatible with the life or well being of the patient. Timidity regarding splenectomy as a means of salvage under such circumstances is being gradually overcome, and the favorable results in our series led us to undertake a critical analysis of 50 patients on whom the operation was performed. This preliminary report deals with the first 24 cases in which the studies are complete.

In most instances "secondary hypersplenism" provided the indication for splenectomy, the patients displaying hemolytic anemia and thrombocytopenic purpura, frequently associated with neutropenia. A few of the spleens were removed because of their bulk or recurring major infarction. The operative risk was not found to be significantly high, although a number of the patients were in exceedingly poor condition and could not be adequately prepared by transfusions because of auto-hemagglutinins. Only one patient died during the immediate postoperative period.

A general evaluation of the results revealed that half of the patients showed satisfactory and occasionally remarkable clinical and hematologic improvement. Some benefit was obtained in 11 others, and only two failed to respond in any fashion. There was no evidence that splenectomy increased the susceptibility of the patients to infection, as has been suggested by some authors. To the contrary, certain ones were quite free of infection for long periods of time, probably by virtue of their improved hematologic status.

SYMPOSIUM ON DISEASES CAUSED BY ENVIRONMENTAL FACTORS (DUST, GASES, AND OTHER PHYSICAL AND CHEMICAL AGENTS)

Referee (by invitation of the Council): Wilhelm C. Hueper

PATHOLOGY OF THYROID TUMORS INDUCED IN SHEEP BY THE PROLONGED DAILY ADMINISTRATION OF I^{131} . Sidney Marks, Lynn A. George, Jr., and Leo K. Bustad, Hanford Laboratories, General Electric Company, Richland, Wash.

In the course of a long-term study on the effects of small quantities of I^{131} administered daily to sheep, four members of a group of five sheep showed multiple thyroid adenomas when sacrificed at an age of 68 months. The fifth member of the group had developed a fibrosarcoma in or about the thyroid gland at 53 months of age. The members of the group had been fed 5 μ c. of I^{131} per day throughout their lives after being exposed to I^{131} *in utero* and during lactation by way of their dams who were also fed 5 μ c. daily.

The number of adenomas in each gland varied from 2 to 13. The histologic patterns of the adenomas were follicular, tubular, or cord-like. The tumors showed partial or complete encapsulation, but extension beyond the capsules occasionally occurred. The cellular appearance was usually uniform although pleomorphism was present in a few tumors. In the fifth animal, the fibrosarcoma completely replaced one lobe of the affected thyroid gland and had metastasized to the lungs.

The amount of I^{131} administered caused mild to moderate histologic damage in the glands, the principal manifestation being interfollicular edema.

No thyroid tumors have occurred in animals fed 0.15 μ c. per day for a similar period nor in any of the control animals.

THE HISTOGENESIS OF OSTEOGENIC SARCOMA AS INDUCED BY RADIOACTIVE CALCIUM AND STRONTIUM. J. F. Kuzma* and Gloria E. Zander, Marquette University, School of Medicine, Milwaukee, Wis.

Development of osteogenic sarcoma was studied in mice and rats given calcium⁴⁵, strontium⁸⁹⁻⁹⁰ intraperitoneally. These radioactive isotopes are bone seekers; that is, the fraction of the administered dose which is retained (roughly 50 per cent) is deposited in the bones and not in soft tissues. Natural mobilization of these isotopes in burdened bone is infinitesimal even over a very long period. These isotopes cause radiation changes in the bones in relationship to their radioactive half-life and the beta energy produced. The changes are best studied in long bones, in the metaphysis, where autoradiograms show the greatest concentration of the radioactive isotope.

The recognizable changes in a rough chronologic order are as follows: (A) Disappearance of cellular bone marrow and osteocytes. (B) Swelling of epiphyseal cartilage cells. (C) "Severance" or detachment of metaphyseal cartilaginous bone from the epiphyseal plate. (D) Migration of old spongiosa toward the shaft with resumption of bony growth after initial retardation. (E) Fibroblastic proliferation in marrow beneath epiphyseal plate. (F) Periosteal fibroblastic proliferation. (G) Differentiation of osteoid in medullary and periosteal "fibroblastic" proliferations. (H) Atypical new bone formation, medullary and periosteal. (I) Thinning and perforation of metaphyseal cortex. (J) Coalescence of medullary and periosteal new bone formation and progress to neoplastic bone growth in various degrees of differentiation. Peripheral portions show least differentiation and all types of osteogenic sarcoma may be represented in one tumor or in one animal. Even synovial sarcoma may be suggested; however, some degree of bone differentiation is present in all tumors.

The patterns and sequence of events described are similar for tumors induced by radioactive calcium and radioactive strontium. However, three differences may be noted: (1) Squamous cell carcinoma of snout region develops in some animals given strontium⁸⁹⁻⁹⁰. This is related to the radiation injury of the epithelium which is closely applied to strontium-burdened bone which, although showing radiation osteitis and repair, has not yet accomplished the development of an osteogenic sarcoma. (2) Radioactive strontium has produced no tumors of the spine or pelvis in these rats. (3) Radioactive calcium produced tumors in the vertebral bodies, pelvis, and especially affected the small bones of the hands and feet. In these areas, periosteal new bone formation is extreme.

The aim of the presentation, therefore, is to show the sequence of histologic events leading to the development of osteogenic sarcoma.

HISTOCHEMICAL STUDIES ON X-IRRADIATED CARTILAGE AND BONE. P. L. Melanotte and Richard H. Follis, Jr.,* Armed Forces Institute of Pathology, Washington, D.C.

Clinical and experimental observations have indicated clearly that x-rays profoundly affect the integrity of epiphyseal cartilage cells and of osteoblasts. Since an understanding of some biochemical processes in normal cartilage and bone has been obtained as a result of application of certain histochemical techniques, these methods have been applied in a study of cartilage and bone to which x-rays of varying dosages have been administered. The right lower extremity of five series of rats weighing approximately 50 gm. was exposed once to 250 kv., 15 ma. x-rays in dosages ranging from 400 to 1800 r. An animal of each group was killed daily thereafter, until all had been sacrificed (10 days post-irradiation). Histochemical studies were carried out on fresh and fixed tissue as follows: cytochrome oxidase, tetrazolium, PAS, alcian blue, toluidine blue, alkaline phosphatase, reticulum. The multiplication and spatial arrangement of the epiphyseal cartilage cells are affected early. Two distinct alterations are found: (1) an irregular deposition of glycogen

in areas where this material is not ordinarily found, i.e., in the resting and early proliferative cell zones, and (2) abnormal distribution of alkaline phosphatase activity in cells and the matrix about them in areas where ordinarily one does not see evidence of enzymatic activity. Such changes are not seen when growth of cartilage is interfered with, for instance by inanition. Perhaps the most striking change is found in the osteoblasts lining the cortex of the shaft of the tibia. Here after 24 hours mitotic figures may be noted in excess of those present normally. Moreover, very early (that is, in the first day) there is proliferation of argyrophilic fibers about these proliferating osteoblasts. Moreover, excessive phosphatase activity is seen in and about the cells, particularly with relation to the reticulum fibers. As time goes on, such fibers coalesce to form a metachromatic-staining matrix which soon shows definite evidence of mineralization. Certain other alterations have been observed which are interpreted as non-specific, resulting from growth suppression.

EXPERIMENTAL MASSIVE PROGRESSIVE PULMONARY FIBROSIS. Paul Gross,* Marian L. Westrick, and James M. McNerney, Mellon Institute, Pittsburgh, Pa.

Massive progressive pulmonary fibrosis designates a peculiar disabling and fatal disease encountered in some British coal miners. It recently has been produced experimentally in guinea pigs in the laboratory of E. J. King, London, with synthetic coal dust (low in silica) combined with an infection of avirulent tubercle bacilli. We are reporting the production of a very similar disease in guinea pigs with a non-fibrogenic silica combined with infection by the Trudeau RIRv strain of avirulent tubercle bacilli.

Infection alone with the RIRv strain of tubercle bacilli in control animals produced relatively small, limited lesions with only minimal focal collagen production. Although the silica dust used consisted of 84 ± 6 per cent alpha quartz and the average particle size was 0.2μ , it failed to produce significant pulmonary fibrosis in control guinea pigs after exposure to adequate concentrations of the dust for 12 months followed by holding periods up to 1 year. The non-fibrogenic character of the silica is attributed to an appreciable (8 per cent) contamination with iron.

HISTOPATHOLOGIC STUDY OF STANNIC OXIDE USED FOR RADIOGRAPHIC VISUALIZATION OF LIVER AND SPLEEN. Harry W. Fischer and William R. Platt,* State University of Iowa College of Medicine, Iowa City, Iowa, and Washington University, School of Medicine, St. Louis, Mo.

Benign pneumoconiosis due to inert inorganic tin oxide has been regarded as a clinical entity since 1944; in the interim a total of 132 cases have been reported. Stannic oxide is radio-opaque and the "benign" inhalation connotation signifies the retention of inert dust in the lymphatics of the lungs without evidence of irritation, allergy, fibrosis, impaired lung function, chest symptoms, or increased susceptibility to tuberculosis or other infections. This experiment was designed to determine whether or not the intravenous administration (not inhalation) of an inert metallic powder (stannic oxide) could serve as an adequate hepatolienographic agent without producing harmful tissue reactions.

Eight white rabbits and four mongrel dogs were injected intravenously with colloidal suspensions of stannic oxide, the particles therein being about 1μ in size. Roentgenologic studies of the chest and abdomen were made and followed by necropsy studies at different intervals after administration of stannic oxide; i.e., after 90 days in the dog and 30 days in the rabbit. Control studies were made also. Roentgenologically, only the liver and spleen were well visualized. The histopathologic changes seen were characterized by marked to moderate deposition of brownish stannic oxide pigment granules in the prominent reticulo-endothelial cells of the spleen and liver. No other concomitant histologic response was observed. Only occasional granules were observed in the lungs and adrenal glands. Since this

was only a short term experiment, further long term experiments are now being projected to ascertain the tissue reaction sequelae of prolonged deposition of stannic oxide in the reticulo-endothelial system proper. Previous pathologic studies in the human inhalation type would seem to indicate that later histopathologic changes, such as fibrosis, are not to be expected.

THE EFFECT OF ATMOSPHERIC IRRITANTS ON CILIARY ACTIVITY AND MUCUS SECRETION OF THE RESPIRATORY TRACT IN RELATION TO THE PATHOGENESIS OF PULMONARY CANCER. Paul Kotin,* Hans L. Falk, and Herta Tremer, University of Southern California School of Medicine, Los Angeles, Calif.

Ciliary activity and mucus secretion are the mechanisms serving to prevent abnormal deposition and prolonged residence of respired particulate phase of atmospheric carcinogenic agents on the respiratory epithelium. The physiologic defenses may be overcome by a large number of ubiquitous irritative substances in the atmosphere.

Carcinogenic polycyclic aromatic hydrocarbons have been recovered from the atmosphere of urban communities. The chemical and physical nature of these atmospheric carcinogenic agents strongly suggests that prolonged cellular contact and extended epithelial residence are necessary prerequisites for any assumed biologic effect.

The modifying effect of atmospheric substances on the normal functions of the tracheobronchial tree has been studied in two ways. First, animals have been exposed in inhalation chambers to controlled test environments for varying periods. After removal, their tracheobronchial trees were exteriorized and opened so that ciliary activity and mucus secretion could be observed in the intact animal. A second method consisted of using isolated tracheobronchial epithelial strips for *in vitro* studies. Carbon particles of a size, configuration, and chemical structure identical to those recovered from the atmosphere were used for the determination of ciliary rate. A stereomicroscope with scaled oculars was adapted for this purpose. In addition, studies were carried out using the upper respiratory-esophageal tract of frogs. An initial period of excitation was manifested by an increased rate in the movement of particles. This was followed by a period of decreased activity which, if allowed to persist, often resulted in total cessation of particle movement. Prolonged or intense exposure was followed by limited and delayed recovery. Total ciliary paralysis could be maintained for a considerable length of time following which partial recovery was still possible. Morphologically, it was possible to demonstrate a considerable degree of parallelism between alterations in physiologic functions and cellular abnormalities.

Mucus secretion was affected also by exposure of the respiratory tract to irritants. Physiologically, alterations were manifested initially by an increase in secretion. Demonstration of this response was noted in microscopic studies of the respiratory epithelium. An increase in the number and activity of goblet cells was the first histologic change noted. This progressed until there was almost complete replacement of the normal epithelium by an overgrowth of these mucus-producing cells. Subsequently, pools or lakes of mucus were formed in the epithelium. Finally, the mucus lakes emptied into the lumen of the tracheobronchial tree with complete epithelial desquamation down to but not including the basal cells.

The assembled data suggest the following conclusions: (1) An apparent purposefully exaggerated activity of the respiratory defense factors is the initial response of the respiratory epithelium to the deposition of foreign particulate substances. (2) Persistence of the insulting agents rapidly leads to a neutralization of the protective factors with resultant slowing of particle movement leading to progressive accumulation of particles at selected sites in the respiratory tract. (3) A demonstrable difference in the effect on the epithelium can be observed,

depending upon whether the respired material directly impinges on or passively flows over tracheobronchial epithelium. (4) Reversibility of the adverse effect of atmospheric environmental agents endures for an unanticipatedly long period; although restoration to baseline levels of activity seldom occurs. (5) The denudation of the superficial epithelium permits the immediate apposition of respired particulate carcinogenic matter to the persisting layer of basal cells.

Alterations in the normal physiologic activity of the respiratory epithelium appear to be significant in the pathogenesis of pulmonary cancer. The persistence of carcinogenic hydrocarbons on the respiratory epithelium provides an opportunity for their elution from atmospheric soot upon which they are adsorbed. Further, denudation of the superficial epithelium juxtaposes the carcinogenic agents to the basal cell layer where presumably the process of cancerization begins.

Of equal significance and interest is the demonstration of an exaggerated, more persistent effect of particulate matter on areas of impingement when contrasted with those of passive flow.

Studies of the epithelial changes in experimental species disclose that an internal environment may be produced which is compatible with the biologic activity of the respired carcinogenic agents. Similarities between the experimental observations and studies on the respiratory epithelium of humans will be discussed.

ASBESTOS: AN EXTRINSIC FACTOR IN THE PATHOGENESIS OF BRONCHOGENIC CARCINOMA. Ward M. O'Donnell* and Richard H. Mann, Lancaster General Hospital, Lancaster, Pa.

During a period of 16 years (1940 to 1956) there were 27 patients with proved pulmonary asbestosis seen at the Lancaster General Hospital. The observations were established by necropsy in 24 cases and by examination of the lungs removed at surgery in 3 patients. In 13 of these individuals there was an associated bronchogenic carcinoma.

The ages of the patients who had both asbestosis and bronchogenic carcinoma ranged from 37 to 69 years with an average age of 54 years. Available information showed the median total number of years of occupational exposure to be 21.5 years. The interval from the initial exposure until the recognition of the neoplasms varied from 20 to 40 years. Although some of the patients were still employed in the asbestos textile mill when evidence of neoplasm appeared, there were several in whom carcinomas developed 20 to 30 years after cessation of exposure, while they were no longer workers in this industry.

The primary anatomical site of the carcinoma was in the lower lobes of the lungs in all cases: 7 involved the lower lobe of the right lung and 6 began in the lower lobe of the left lung. In the morphologic classifications of the neoplasms, 8 were found to be of the squamous cell type, 2 were anaplastic, and 3 were adenocarcinomas.

This frequent association of pulmonary asbestosis and bronchogenic carcinoma (almost 50 per cent) as seen in this study lends further support to the proposition that asbestos is a carcinogen in susceptible individuals after critical exposure in the textile phase of the industry where asbestos dust is in high concentration.

EFFECT OF METHYLCHOLANTHRENE ON THE TRACHEA OF WHITE PEKIN DUCKS. R. H. Rigdon,* Medical Branch, University of Texas, Galveston, Texas.

Methylcholanthrene, when applied to the skin of ducks daily for 30 days, produced papillomas, fibromas, hemangiomas, neurofibromas, and ganglioneuromas within a period of 3 to 6 months. In the present study methylcholanthrene in mineral oil (1.5 gm. per 100 ml.) was put into the trachea through the external larynx; 0.5 ml. was given daily for as long as 26 days. Some of the ducks were

observed for as long as 1 year thereafter while others were sacrificed at more frequent intervals for pathologic study. Many of the specimens were observed under ultraviolet light for the presence of a fluorescence substance consistent with methylcholanthrene. A total of 55 ducks had methylcholanthrene in mineral oil put down their trachea, 26 had only mineral oil, and 95 were used as controls. Metaplasia in the epithelial cells of the trachea was a frequent finding but no tumors occurred.

CELLULAR LOCALIZATION OF FLUORESCENT PRODUCTS DERIVED FROM CIGARETTE SMOKE: STUDIES IN EXPERIMENTAL ANIMALS AND IN MAN BY FLUORESCENCE MICROSCOPY.† Robert C. Mellors,* Sloan-Kettering Institute for Cancer Research, New York, N.Y.

Cognizant of epidemiologic data pertaining to the possible relation between the degree and duration of the tobacco-smoking habit and the incidence of carcinoma of lung, larynx, and oral cavity, and of experimental data indicating that cigarette "tars" (smoke-condensates) are active in epidermal carcinogenesis in some species of laboratory animals, we have undertaken the microscopic study of the cellular localization *in vivo* of fluorescent products derived from cigarette smoke. Of course, the chemical composition and the biologic activity of the materials was not to be inferred from fluorescence.

It was found that when the skin of mice was painted with whole cigarette tars and chemical fractions thereof known to be active in experimental carcinogenesis, the microscopic pattern of cutaneous localization of the fluorescent materials in these tars was comparable to that observed in early studies of a similar kind with the carcinogens, benzpyrene and 20-methylcholanthrene. The active cigarette tars displayed an affinity for lipids and for keratin in the skin; carcinogenically inactive fractions of cigarette tar did not so localize.

A study of the smoking effects on buccal epithelium was carried out on normal human smokers and on patients (in collaboration with Charles Harrold) with leukoplakia of the oral cavity. It was found that fluorescent materials in cigarette smoke localized preferentially in keratinized and lipid-containing epithelial cells of the buccal mucosa, especially in patients with leukoplakia.

BOVINE HYPERKERATOSIS CAUSED BY CHLORINATED NAPHTHALENES. Carl Olson, University of Wisconsin Medical School, Madison, Wis.

From 1941 to 1953 a serious disease affected herds of cattle in nearly all parts of the United States. During this interval it occurred with increasing frequency. The disease was characterized by a progressive hyperkeratosis, a proliferative stomatitis, emaciation, reproductive disturbances, and death with secondary bacterial infections. Many theories on etiology were extant. Several laboratories of animal pathology became involved with the problem and in 1949 a program of coordination of work was begun. Soon thereafter feedstuff was incriminated as the cause. This included pelleted feeds, bread crumbs from a bakery, and hay from a specific meadow. Exposure to a food preservative also was found to cause the disease. Lubricants of farm machinery were next incriminated, which provided the clue for solution of the problem.

The pathology of chlorinated naphthalene poisoning in cattle is somewhat different from that of man or laboratory animals. The proliferative stomatitis associated with bovine hyperkeratosis is due to a virus which is without effect in adult animals not poisoned with chlorinated naphthalene. The use of non-toxic lubricants in feed processing machinery has practically eliminated the disease.

† This work was aided by a grant from the American Cancer Society, Inc.

PHYSIOPATHOLOGY OF COLD INJURY: GANGRENE OF THE EAR PRODUCED IN MICE BY EXPOSURE TO LOW ENVIRONMENTAL TEMPERATURES (1° TO 5° C.).† J. P. Kulka,* R. A. Wolbach, and G. J. Dammin,* Harvard Medical School and Peter Bent Brigham Hospital, Boston, Mass.

Cold-induced gangrene without prior tissue freezing is known to occur in man but has been observed only incidentally in experimental animals. We have found that gangrene of the ears develops with regularity in white mice kept in individual cages at 1° to 5° C. for 7 to 10 days.

The mice were observed daily and the ears were studied histologically after sacrifice at the end of 3, 5, 7, and 10 days in the cold and at intervals up to 3 weeks after these periods of exposure. During the first day of exposure the ears usually became blanched. Subsequently, regions of blanching persisted in the distal portions and erythema developed proximally. After 3 to 5 days in the cold, inflammation became apparent in circumscribed regions corresponding to terminal vascular beds, particularly at the anterior and posterior margins of the distal pinnae. Evans blue, injected intravenously after the animals were returned to room temperature, accumulated in these regions, indicating increased endothelial permeability.

The following microscopic changes occurred in approximate chronologic order: edema, focal necrosis of sebaceous glands, slight to moderate neutrophilic infiltration, segmental necrotizing angiitis involving venules more extensively than arterioles, focal interstitial fibrin deposition, focal extravasation of red blood cells, vascular obstruction by leukocytic thrombi, patchy epidermal necrosis, focal necrosis of skeletal muscle, focal necrosis of adipose tissue, fixed-cell proliferation, and gangrene accompanied by massive fibrin thrombosis. The gangrene usually developed first at the inner surface of the anterior margin of the pinnae and initially spared the tips of the ears which are supplied directly by the central arteries. Focal necrosis of blood vessels, skeletal muscle, and adipose tissue occurred even in proximal regions where the epidermis remained intact.

The cold-induced histologic changes observed in the ears of mice resembled those of frostbite as well as trench foot in man. This finding supports the concept that the pathogenetic mechanisms of clinical cold injury at temperatures below and above freezing are similar. A characteristic feature of both the human and the experimental lesions is the focal necrosis of blood vessels, sweat glands, fat, and skeletal muscle. This pattern of cell damage is difficult to explain either on the basis of direct injury by the diffuse chilling or indirect injury by patchy ischemia. The onset of gangrene in circumscribed regions at the distal margins of the ears appears to follow an increase in endothelial permeability in these regions and suggests that circulatory impairment, possibly vasospastic in nature, determines the extent of permanent tissue loss.

THE PATHOLOGIC SIGNIFICANCE OF THE NEW ENVIRONMENTAL DISEASE PANORAMA RESULTING FROM MODERN INDUSTRIALIZATION. Wilhelm C. Hueper* (Referee‡), National Institutes of Health, Bethesda, Md.

* * *

† Supported by Contract DA-49-007-MD-645 (Research and Development Division, USG, DA) and Grant H-2206 from the National Heart Institute, National Institutes of Health, United States Public Health Service.

‡ By special invitation of the Council.

THE STRUCTURE AND COMPOSITION OF LUPUS BODIES. Boris Gueft,* Veterans Administration Hospital and University of Cincinnati College of Medicine, Cincinnati, Ohio.

Previous investigations of the L.E. cells and hematoxylin bodies of systemic lupus by Klemperer and his associates have emphasized their nuclear origin and an unusual alteration or "depolymerization" of the nucleic acids found in these structures. Godman, however, has pointed out that the proof of "depolymerization" depended on weak methyl green staining of these masses and that this weak staining was due more to interference by protein rather than any other change in the desoxyribose nucleic acid (DNA). Further studies of the alteration in the L.E. body have been performed, therefore, by electron and ultraviolet microscopy and by quantitative microspectrophotometry of protein content.

Ultraviolet microscopy and spectrophotometry reveal that the L.E. cell has a marked broad absorption peak at 2700 Å, hitherto unreported. This finding, together with the positive Feulgen reaction, demonstrates that DNA is present in the mass. Measurement shows that the DNA amount is roughly equal to that normally found in any human cell. Measurements of the Millon reaction according to Pollister and Mirsky's technique indicate that more protein is present per body than is present in a normal lymphocyte nucleus. Electron microscopic observations have shown that in some instances the L.E. body retains a semblance of the cell from which it arose to a greater extent than one can judge by conventional microscopy. The nucleus, nucleolus, and a thin rim of material that may be cytoplasm are seen in the electron photomicrograph. No "virus" or other particles are found.

Fluorescence microscopy points to the possibility that a component foreign to the normal cell is present in the L.E. body, perhaps protein, judging from the slight yellow-white fluorescence. The positive PAS stain of the bodies also indicates a polysaccharide of unknown origin. Even fibrin cannot be ruled out.

These findings suggest that the cytoplasm of the degenerating lymphocyte or polymorphonuclear leukocyte may participate in the formation of the L. E. body. Moreover, it appears that a plasma protein may be present, and that these components may account for the high concentration of protein previously found in L.E. inclusions and in "hematoxylin" staining bodies.

FURTHER PATHOGENETIC STUDIES OF DISEASES OF UNKNOWN ETIOLOGY, WITH PARTICULAR REFERENCE TO DISSEMINATED LUPUS ERYTHEMATOSUS AND BOECK'S SARCOID.† Robert C. Mellors,* Louis G. Ortega, Wilbur F. Noyes, and Halsted R. Holman, Sloan-Kettering Institute for Cancer Research and Rockefeller Institute for Medical Research, New York, N.Y.

In previous work we reported that gamma globulins were localized in the glomerular lesions in human (and experimental) glomerulonephritis, lipoid nephrosis, polyarteritis nodosa, and amyloidosis but were not similarly found in normal kidneys or in kidneys the sites of a variety of common infectious, inflammatory, degenerative, vascular, and neoplastic abnormalities. In continued studies we utilized fluorescein-labeled antibodies and suitable control reactions for the detection of the following antigens: gamma globulins; other serum proteins; C-reactive protein; streptolysin O; and somatic antigens of streptococci, group A, types 12 and 4.

In chronic membranous glomerulonephritis (type 2 of Ellis, with the nephrotic syndrome) gamma globulins, but not detectable quantities of other serum proteins, are present in the thickened glomerular basement membranes. In disseminated lupus erythematosus gamma globulins are localized strikingly in the "wire-loop" lesions and in thickened (and possibly doubled) basement membranes of involved

† Supported by a grant from the National Heart Institute, National Institutes of Health, United States Public Health Service.

glomeruli, occasionally in focal areas of Bowman's capsule, in some plasma cells in the interstitium, and rarely in sites of hyaline droplet degeneration in tubular epithelium. In disseminated lupus erythematosus, C-reactive protein is focally localized in renal tubular epithelium and in some glomeruli, in a pattern dissimilar from that of gamma globulin localization. In Boeck's sarcoid gamma globulins are localized in the lymph nodes, not in the epithelioid granulomas but in their immediate surroundings in which are present hyaline materials (paramyloid) and plasma, lymphoid and reticular cells.

The foregoing findings will be viewed in several respects and in the light of Teilum's statement: "In comparative pathologic-anatomic studies of Boeck's sarcoid, lupus erythematosus disseminatus, and other conditions, I have demonstrated, as a common feature in such disorders of different causation, a coincidence of hyperglobulinemia, paramyloidosis, or hyalinosis in the reticulo-endothelial system which is often decisive as a morphologic specific character, and is sometimes found in direct relation to an accumulation of plasma cells. . . . According to my observations, these changes must be considered phases of *an elementary morphologic immunity reaction with an underlying allergic hyperglobulinosis in the reticulo-endothelial system.*"

DEMONSTRATION OF SPECIFIC ANTIGEN AND ANTIBODY IN EXPERIMENTALLY PRODUCED AMYLOID. Jacinto J. Vazquez, Frank J. Dixon,* and Alexander L. Neil, University of Pittsburgh, School of Medicine, Pittsburgh, Pa.

Our previous finding of concentrations of homologous gamma globulin in the amyloid deposits of secondary human and experimental amyloidosis was consistent with the possibility that such gamma globulin represented antibody. The present study with the fluorescent technique of Coons *et al.* demonstrates that amyloid produced by the injection of casein does contain specific antibody and antigen.

Amyloid deposits in spleen were produced in the rabbit by repeated subcutaneous injections of casein (sodium caseinate). Specific rabbit anti-casein was obtained by immunization with the same material used to produce amyloid in the rabbit. The globulin fraction of this anti-casein serum was labelled with fluorescein isocyanate and served as an immunohistochemical stain to detect the presence of the corresponding antigen and/or antibody in the amyloid deposits. It was found that such deposits contained appreciable concentrations of specific antibody (anti-casein) and to a lesser degree specific antigen (casein). The localization of specific antibody corresponded with that of homologous gamma globulin. Parallel studies of similar amyloid deposits produced after repeated injections of ribose nucleic acid served as negative biologic controls for the above observations. It is concluded that, under the conditions of our experiment, the amyloid deposits in the spleen of experimentally produced casein amyloidosis contain specific antibody and to a lesser degree antigen. These findings suggest that the deposition of antigen and antibody may be one mechanism for the formation of amyloid.

CELLULAR SITES OF FORMATION OF GAMMA GLOBULIN.† Louis G. Ortega and Robert C. Mellors,* Sloan-Kettering Institute for Cancer Research, New York, N.Y.

Numerous observations have established the dominant rôle of lymphatic tissue and, especially, its plasma cell content in the formation of gamma globulin. Nevertheless, many unresolved problems remain that appear suitable for analysis by fluorescent antibody technique. In our investigation of the subject we have used

† Supported by a grant from the National Cancer Institute, United States Public Health Service.

fluorescent antibodies prepared against human gamma globulin to determine the distribution of the latter in the plasmacellular and lymphoid aggregates of a variety of lesions and reactive tissues in humans. Throughout this work, the fluorescein-conjugated antibody "stain," appropriately absorbed with tissue powders, was applied to fresh, unfixed, washed frozen sections. The immunologic specificity of the resulting staining patterns was evaluated by the Coons "blocking" procedure. The staining attributed to the presence of localized gamma globulin was in each case that which could be abolished by prior reaction of the conjugate with highly purified human gamma globulin but which was unaffected by prior reaction with other serum proteins, principally albumin.

The human tissues thus far examined include: "normal" and minimally to moderately hyperplastic lymph nodes and spleens, spontaneous human cancers, homotransplanted human cancers, and lesions of certain connective tissue diseases.

In the present report, attention has been directed solely at the apparent cellular sites of gamma globulin formation without reference to the specific localization of gamma globulin in other areas or to the role of antigenic stimuli.

Fluorescence analysis revealed gamma globulin in the cytoplasm of three distinct, readily identifiable cell types and in certain other much less frequent forms as yet unclassified. The identified types were: (1) immature and mature plasma cells with moderate to intense, smooth or granular, cytoplasmic staining for gamma globulin. The cytoplasm of these cells was basophilic in hematoxylin and eosin control sections. (2) Plasma cells containing globular cytoplasmic bodies having a shell-like deposit of intensely stained gamma globulin on their outer surfaces. These corresponded in hematoxylin and eosin and PAS-stained control sections to plasma cells containing Russell bodies of varying maturity. (3) Certain cells of the germinal center of lymph follicles having large nuclei and scanty, finely granular cytoplasm with stellate cytoplasmic processes. These cells contained gamma globulin in the cytoplasmic granules only and we have classified them as primitive reticular cells. The distribution and significance of these cells will be discussed.

CELLULAR AND INTERCELLULAR REACTIONS IN EXPERIMENTALLY ALLERGIC ARTHRITIS AND CARDITIS. Henry Z. Movat and Robert H. More,* Queen's University, Faculty of Medicine, Kingston, Ont.

The experiments to be reported deal with the injury, reactivity, and repair of the articular and cardiovascular connective tissue of the rabbit in local and systemic sensitization.

Four experiments were carried out. In the first, the Arthus phenomenon was elicited in the knee joint with a single dose of horse serum in a hypersensitive rabbit; in the second, repeated intra-articular injections were given over several months; in the third, and in the fourth one, three massive intravenous doses of horse serum were given. The joints and the spleens were examined at serial time intervals in all experiments, and the hearts in the last two.

Although these lesions differ from one another, they have one feature in common: the injury, reactivity, and repair of connective tissue. The injury is characterized mainly by exudative phenomena (serous, serofibrinous, and fibrinous inflammation), the reactivity by mononuclear proliferation (macrophage and plasma cellular reaction), and the repair by organization of the exudate and of the damaged connective tissue.

Conspicuous changes are the formation of mucinous edema and of fibrinoid. The former results from an edematous swelling (serous inflammation) of acid-mucopolysaccharide-rich connective tissue. The latter was found to derive from exuded fibrin. Histochemical and histo-enzymatic studies support this view. An-

other conspicuous change is the proliferation of plasma cells, not described by previous investigators. This plasma cellular reaction in the joint lesion is paralleled by a similar cellular reaction in the spleen.

Allergic arthritis, carditis, and allergic (hypersensitivity) reactions in general are not specific, nor is fibrinoid formation specific for allergic injury, since we could produce the latter also by mechanical, chemical and radiation injury. However, hypersensitivity reactions are characterized by three components: the acute exudative component, including at times fibrinoid; the intense macrophage reaction (which is not consonant with the intense exudative reaction); and the plasma cellular reaction. The function of the macrophages is probably that of removing antigen, whereas the plasma cells represent the morphologic counterpart of antibody formation against the specific antigen. The plasma cells provide morphologic evidence that the tissue injury is centered around some aspect of a local immune reaction.

DIFFERENCES IN THE LOCALIZATION OF A BACTERIAL POLYSACCHARIDE IN LABORATORY ANIMALS. Russell S. Jones* and Yolande C. Mayne, University of Utah College of Medicine, Salt Lake City, Utah.

Klebsiella pneumoniae bacterial polysaccharides have been labelled through biosynthesis with C^{14} . After parenteral injection into the guinea pig, rat, mouse, and rabbit, the polysaccharide is localized in tissues by isotope-tracer, autoradiographic and immunologic techniques. The most striking difference is in the adrenal gland and lymph nodes of the four species. In the guinea pig the highest concentration of C^{14} is in the adrenal gland, in contrast to the minor amount in the adrenal glands of the rat, mouse, and rabbit. In the rat, mouse, and rabbit, the highest concentration of polysaccharide is in the lymph node, while there is little localization in the lymph nodes of guinea pigs. Strip film and contact autoradiographs of tissues from the guinea pig and rat reveal localization within plasmacytes and reticulo-endothelial cells. In cortisone-treated rats the C^{14} content of nodes is still very high even though the lymph nodes consist only of some reticulo-endothelial cells. In the cortisone-treated guinea pigs, there is but slight decrease in C^{14} content and little histologic change of the lymph nodes. The spleen of the rat shows the greatest concentration about but not within the lymph follicles while in the guinea pig the C^{14} is uniformly distributed in the splenic pulp and is not present in the lymph follicles. The bacterial polysaccharides produce an acute arthritic lesion in the guinea pig and autoradiographs show intense localization in the synovium. Arthritic lesions do not occur in the rat and little C^{14} localization is noted in the scanty synovial cells of their joints.

SPECIFIC IMMUNOCHEMICAL STAINING OF CRYPTOCOCCUS NEOFORMANS AND ITS POLYSACCHARIDE IN TISSUE. Warren C. Eveland, John D. Marshall, Arthur M. Silverstein, Frank B. Johnson, Lalla Iverson,* and Donald J. Winslow, Armed Forces Institute of Pathology, Washington, D.C.

The fluorescent-labelled antibody technique of Coons and coworkers has been applied to the specific immuno-histochemical staining of a number of protein and polysaccharide antigens in tissue. We have employed this method in a study of the distribution of *Cryptococcus neoformans* and of its polysaccharide breakdown products in routine formalin-fixed tissues from the collection of the Armed Forces Institute of Pathology. The presence of the specific organisms in various tissues may be readily demonstrated. The specificity of the staining reaction has permitted identification of homologous polysaccharide material in the tubules and interstitial tissue of the kidney as of cryptococcal origin, in the absence of intact organisms in the same area. Similarly, polysaccharide was found lining bronchial epithelium, in alveolar exudate, and within macrophages participating in the granulomatous

reaction. The ease and specificity of the technique suggest its application to a detailed study of the localization of the organism in the host, and of the distribution of polysaccharide material derived from the organism.

OBSERVATIONS ON THE EFFECT OF TUBERCLE BACILLI ON MONOCYTES OF GUINEA PIGS IN TISSUE CULTURE IN A STUDY OF RESISTANCE TO TUBERCULOSIS. Morgan Berthrong,* Glockner-Penrose Hospital, Colorado Springs, Colo.

The mechanism of resistance to tuberculosis has never been clearly elucidated. Antibodies have not been demonstrated to function in either native or acquired resistance. Attention has been directed repeatedly to a possible cellular resistance. From certain animal experiments, it has been thought that monocytes from immunized animals were better able to withstand the injurious effects of intracellular tubercle bacilli and to inhibit the multiplication of such organisms. Furthermore, by methods of tissue culture, recent workers have reached similar conclusions. Yet other experimenters, who have used similar techniques of tissue culture, have been quite unable to demonstrate such activity in the monocytes of immunized animals.

Because of the basic importance of the question, we have studied the problem using certain modified procedures in tissue culture. Monocytes from normal, from sensitized, from immunized and sensitized, and from immunized and desensitized guinea pigs have been obtained from the peritoneal cavity and have been infected with virulent living and killed tubercle bacilli. These have been cultured on side wall and coverslip preparations in roller tubes. The monocytes have been cultivated in both normal and in immunized guinea pig serum as the culture medium. This technique, as with all tissue culture methods for monocytes, has a major defect in a study of a chronic disease such as tuberculosis in that the monocytes survive only for 2 or 3 weeks, whether infected or not. Nevertheless, many pertinent observations on the rate of intracellular multiplication of the bacilli in the different cells and on the effects of the intracellular bacilli upon the behavior of the monocytes have been made.

LEUKOPENIA AND EXPERIMENTAL MUCORMYCOSIS (RHIZOPUS ORYZAE INFECTION). Heinz Bauer and Walter H. Sheldon,* Emory University School of Medicine, Emory University, Ga.

Significant lesions of mucormycosis can be produced only in metabolically abnormal animals. In previous experiments the leukocytes in the fungal lesions of rabbits with either acute alloxan diabetes or infusion hyperglycemia showed nuclear pyknosis and karyorrhexis while the lesions of cortisone treated animals revealed a decreased leukocytic response. In view of these observations, the importance of the leukocyte in experimental mucormycosis was studied in the following experiments by observing the course of this infection in metabolically normal but severely leukopenic animals.

Sustained leukopenia with granulocytopenia (average daily total white blood cell counts from 890 to 1,120 per cmm. with 1.5 to 6.5 per cent neutrophils) was produced in 37 rabbits by repeated intravenous injections of nitrogen mustard. A standardized spore suspension of *Rhizopus oryzae* was then instilled intranasally in 25 animals and normal saline solution in 12. Leukopenia was maintained for 2 to 11 days following inoculation.

Group I consisted of 13 rabbits sacrificed 2 days after inoculation. Groups II and III each consisted of 6 animals which were sacrificed 3 to 7 and 8 to 11 days after inoculation. Group IV comprised the 12 controls sacrificed 2 to 11 days after instillation of saline solution.

Nasal lesions occurred in all rabbits of group I, in four of group II, and in five animals of group III. One rabbit in group II and another in group III showed slight pulmonary involvement. Minimal focal meningitis was present in one rabbit

in each of groups II and III. No lesions were found in group IV. Positive fungal cultures were obtained at necropsy from all animals except one in group I and another in group III.

During the first 2 days following inoculation extensive lesions developed at the site of inoculation which spread by vascular invasion to the deeper tissues of the nose and rarely to the meninges. After 4 days, the lesions became circumscribed, vascular invasion ceased, and the fungus began to degenerate. From the fifth to the eleventh day, the lesions showed progressive repair with an increasing tendency to heal.

Our findings show that leukopenia with granulocytopenia decreases host resistance during the early phases of experimental mucormycosis. During the later phases, however, the lesions regress and host resistance appears unimpaired. The arrest of the lesions suggests that none of the biochemical alterations occurred in the host which in previous experiments were shown to be essential in the pathogenesis of this fungal infection. Therefore, the virtual absence of the leukocytic response in these animals seems to alter only the initial stages of mucormycosis.

EFFECTS OF CORTISONE, TOTAL BODY IRRADIATION, AND NITROGEN MUSTARD ON CHRONIC, LATENT TOXOPLASMOSIS.† J. K. Frenkel,* University of Kansas School of Medicine, Kansas City, Kans.

Numerous cases of active clinical toxoplasmosis have been described in man and animals, in this country and elsewhere. In addition, the prevalence of Toxoplasma antibody and skin sensitivity in seemingly healthy populations attests to the widespread occurrence of inapparent infection. Experimental evidence indicates that asymptomatic infection is frequently followed by chronicity, which may either be latent or symptomatic. The occurrence of a significantly increased incidence of positive tests for toxoplasmosis in patients with chronic recurrent retinochoroiditis indicates that chronic Toxoplasma infection occurs in man likewise, and that it accounts for approximately one half of these cases in the United States. The widespread use of anti-inflammatory corticoids in patients with ocular and other diseases suggested the desirability of information concerning the possible immunity-depressant action of corticoids during chronic toxoplasmosis. The observation of a patient with Hodgkin's disease, who was treated with cortisone, nitrogen mustard, and x-irradiation, and who died from toxoplasmic encephalitis, suggesting relapse rather than primary infection, extended this interest to other agents that are potential depressants of immunity.

About 100 golden hamsters were infected subcutaneously with the RH strain of Toxoplasma. They were allowed to develop immunity while being treated with sulfadiazine; they were then re-infected, re-treated, and finally they were re-infected and proved immune. One month later the surviving animals were divided into groups of 8 to 20 to be treated weekly with cortisone acetate (2.5 mg. subcutaneously), total body irradiation (100 r., 250 kv., 15 ma., hvl, 2.6), or nitrogen mustard (0.1 to 0.4 mg. as tolerated, intraperitoneally), or with combinations of these drugs. At the time of death, selected tissues were titrated in mice for their content of Toxoplasma, and 93 per cent of 73 hamsters were found to have remained infected. However, as determined by complete necropsy and sectioning, death was due to a variety of causes, including anemia, bacterial infection, aortic rupture, and reactivated toxoplasmosis. The results indicate that in chronic toxoplasmosis of hamsters, only cortisone in combination with irradiation, in the doses used, increased the incidence of significantly depressed acquired immunity, which

† Supported by Research Grant E-989 from the National Institute of Allergy and Infectious Diseases, United States Public Health Service.

permits proliferation of *Toxoplasma*, causing fatal encephalitis or pneumonia. Active retinochoroiditis was present in five instances. The types of lesions and the mechanisms of their production will be discussed.

EFFECT OF IONIZING RADIATION ON INFECTIVITY OF CYSTICERCERCI OF *HYMENOLEPIS DIMINUTA*. J. B. Villella, S. E. Gould,* and H. J. Gomberg, University of Michigan, Ann Arbor, Mich.; Wayne County General Hospital, Eloise, Mich.; and Wayne State University College of Medicine, Detroit, Mich.

This study was undertaken in connection with our inquiry on the effect of ionizing radiation on certain food-borne parasites. We could not test the effect of radiation on the cystic stage of the beef tapeworm (*Taenia saginata*) or the pork tapeworm (*Taenia solium*) because man is the only definitive host of each of these tapeworms. We therefore chose the rat tapeworm, *Hymenolepis diminuta*, since its cysticerci can be fed to laboratory animals.

A supply of cysticerci of *H. diminuta* was obtained by feeding portions of mature proglottides containing infective eggs to the flour beetle, *Tribolium confusum*. After 2 weeks, infective cysticerci were recovered from the body cavity of the beetles. Counted numbers (usually 15) of cysticerci exposed to various doses of cobalt-60 or x-ray or non-irradiated cysticerci (controls) were fed by pipette to white rats. The animals were killed after 12 days, the intestinal tract examined, and the number of adult worms counted. Control rats included those fed no cysticerci.

A dose of 15,000 r. x-ray (80, 120, or 245 kv.) or of cobalt-60 applied to the cysticerci prevented infection. Experiments are under way to determine the infectivity of the proglottides of tapeworms that developed from cysticerci which were irradiated.

The practical significance of these results are discussed with respect to irradiation of pork infected with *Cysticercus cellulosae* and beef infected with *Cysticercus bovis* in the control of these tapeworm diseases.

EXPERIMENTAL OSTEO-ARTHRITIS IN RATS PRODUCED BY INFECTION WITH *STREPTOBACILLUS MONILIFORMIS*. Edwin M. Lerner, II* and Emanuel Silverstein, National Institutes of Health, Bethesda, Md.

Several strains of *Streptobacillus moniliformis* have been isolated and characterized from naturally occurring infections of the lung and middle ear in old laboratory rats, and a number of these strains have been adapted to grow on relatively simplified culture media. Most of the strains have appeared avirulent for rats, but one recent isolate has been demonstrated to be pathogenic. After intravenous injection with this strain, young rats developed redness, swelling, and tenderness of one or more joints within 5 to 7 days, which subsided 10 to 30 days after the acute phase in most animals. Radiocarpal, tibiotarsal, and phalangeal regions were affected, occasionally in migratory fashion, and frequently two or more joints were affected at once. Histologically, the lesions indicated arthritis, periarthritis, osteomyelitis, and periostitis. The osteo-arthritis was characterized by an acute inflammatory reaction at the 6th day, subacute to chronic at the 17th day, and chronic fibrotic and proliferative at the 33rd day. Microscopic evidence of infection was noted long after grossly visible swelling and redness had disappeared, and at times was noted in joints which had never shown gross changes. No lesions other than those of bones and joints were observed grossly or microscopically.

Joint lesions could be produced consistently in extremely high incidence by intravenous injection of this strain of *Streptobacillus moniliformis*, but not by subcutaneous injection of the same cultures. The infecting microorganism was recovered from joints ground and cultured at necropsy in a very high proportion

of animals at the acute phase of the disease, although only one blood culture from such rats was positive. Some degree of immunologic response has been demonstrated in infected animals.

This reproducible, high-incidence infectious osteo-arthritis of rats apparently affects bones, joints, and periarticular tissues to the exclusion of all other organs and tissues. *Streptobacillus moniliformis* has been regarded as a commensal micro-organism of low virulence for the rat, and has not previously been demonstrated to be pathogenic for this animal.

THE DISTRIBUTION OF ACID MUCOPOLYSACCHARIDES IN NORMAL KIDNEYS, AS SHOWN BY THE ALCIAN BLUE-FEULGEN (AB-F) AND ALCIAN BLUE-PERIODIC ACID-SCHIFF (AB-PAS) STAINS.† Robert W. Mowry* and Jean C. Morard, University of Alabama Medical Center, Birmingham, Ala.

The distribution of acid mucopolysaccharides in the kidney is not established. Previous studies were limited by difficulties attending the use of toluidine blue metachromasia or dialyzed iron, especially when the amounts of acid mucopolysaccharide are small. While the coloration of acid mucopolysaccharides depends on acidic groups without strict specificity for carbohydrates, the Alcian blue (AB) stain is superior to traditional methods. Acid mucopolysaccharides containing sulfate and/or carboxyl groups are well colored by AB, without coloring ribonucleic acid to any extent.

Human urine contains small but measurable amounts of both acid and neutral polysaccharides, presumably associated with protein as mucoproteins. The dominant mucopolysaccharide is acidic and resembles chondroitin sulfate (CSA). Moerner, Oliver, and others believed the CSA-like substance promoted precipitation of protein in renal tubules. Rubin and Howard, Boyce, and others found that the calcifiable matrix of many renal stones is rich in mucopolysaccharides. Engel and others postulated that both urinary and serum mucoproteins may be derived from the breakdown of ground substance in systemic connective tissues. Little is known concerning urinary mucoproteins in laboratory animals.

As urinary mucoproteins probably originate in the kidney, we have examined the kidneys of rabbits, guinea pigs, rats, and mice for histologically demonstrable acid mucopolysaccharides. Five healthy adults of each sex were examined from each species. Tissues were fixed in neutral buffered formalin and paraffin sections stained by the Alcian blue-Feulgen (AB-F), AB-PAS, and PAS procedures. After the AB-F stain, tissue sections were dipped in saturated aqueous picric acid for 1 minute, rinsed briefly, and then dehydrated.

Descriptions apply to sections stained by the AB-F with picric unless otherwise stated. AB-stained material was found in the ground substance of connective tissues, mast cells, and in the renal tubules. All species showed definite though only moderate coloration of basement membranes of glomeruli and, to a less extent, the renal tubules. In AB-PAS stained sections, the acidic component of glomerular basement membranes appeared outside the membranous component colored by the PAS. There was acidic material, presumably mucopolysaccharide, coating the renal epithelial cells in some segment of the distal tubules or collecting system in each species. Its exact distribution varied with the species but appeared constant in any given species, regardless of sex. A slight to moderate amount of acidic material coating the luminal surface of the macula densa was almost constant in the rabbit and guinea pig but inconstant in the rat and mouse.

The greatest amount of acidic material in renal tubules was found in the guinea pig, viz., coating the surface and in the subjacent cytoplasm of cells lining

† Aided by a grant from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service.

the collecting tubules. Slight amounts were seen coating the cells of the distal convoluted tubules in the rabbit and guinea pig. Traces of acidic material coating the upper or terminal parts of Henle's broad limbs were fairly constant in the guinea pig but inconstant in the rabbit. In renal tubules of the rat, acidic material was largely confined to the surface of cells lining the ducts of Bellini and the terminal part of collecting tubules. Renal tubules of the mouse contained only scant acidic material and this coated the cells of the macula densa about one third of the time in each specimen. Other histochemical properties, the nature, and the possible significance of the acidic substance(s) in the nephron will be discussed.

HISTOLOGIC AND HISTOCHEMICAL CHANGES IN THE KIDNEYS OF RATS FED A DIET WITH AN EXCESS OF INORGANIC PHOSPHATE.† John M. Craig,* Children's Hospital and Harvard Medical School, and Children's Cancer Research Foundation, Boston, Mass.

Young rats of the Sprague-Dawley strain, weighing 60 to 150 gm., with appropriate controls were placed on a chow diet containing 10 per cent added disodium acid phosphate for periods of 24 to 72 hours. Some animals were sacrificed at the end of the feeding period; others were again placed on the control diet for recovery periods of 2 to 7 days. The animals on the experimental diet maintained their weight, but developed polydipsia and polyuria which persisted even after returning to a normal diet. At necropsy the kidneys were enlarged, the degree of enlargement correlating with the observed food intake.

Lesions developed in the inner cortex, outer medulla, and less frequently in the outer cortex. These lay chiefly in the distal portion of the proximal convoluted tubule, the ascending limb of Henle's loop, the distal convoluted tubule, and the collecting tubule and papillae. Micro-incineration techniques and Bunting's phosphate stain revealed marked deposition of mineral in many structures not recognized by ordinary techniques in the cortex and medulla. Changes in the usual distribution of succinic dehydrogenase, esterase, and acid phosphatase were observed, though no change in alkaline phosphatase activity was found.

EXPERIMENTAL STUDIES OF RENAL FUNCTION IN ACUTE URETERAL OBSTRUCTION. R. Dominguez,* Robert B. Adams, and Frank MacIntyre, St. Luke's Hospital, Cleveland, Ohio.

We have studied, in heparinized dogs under nembutal narcosis, the renal excretion during acute unilateral ureteral obstruction and in the first few hours following release of the obstruction. Renal excretion was tested by the constant injection of radioactive diodrast. The radioactivity of the blood was monitored continuously by an arteriovenous shunt coiled around a crystal scintillation counter. After equilibrium between blood and other body fluids had been established (1 to 2 hours), the constancy of the blood level means that the rate of excretion is equal to the rate of injection.

Urine was collected at suitable intervals, from the right kidney during the whole experiment and from the left during unobstructed periods. At the end of the experiment, the gastro-intestinal organs, spleen, diaphragm, and kidneys were wet-ashed and assayed for radioactivity.

Diodrast was injected either intravenously or into the left ureter near the renal pelvis. In some instances, the pressure developed within the pelvis was measured and recorded. In some experiments the release of the obstruction was sudden and in others gradual. In a group of experiments with intravenous diodrast, the renal occlusion was produced by the intrapelvic infusion of saline solution under controlled flow and pressure. From the results of the experiments the

† Supported by United States Public Health Service Grant C-1975 (C₃).

following conclusions have been reached: (1) The return of diodrast from the kidney to the blood (pyelovenous backflow) depends on the pressure developed within the pelvis and may be quite considerable at the higher pressures produced (160 mm. of Hg). (2) The kidney continues to excrete diodrast for several hours of complete ureteral obstruction without sign of impairment. The gradual reduction of the obstruction ($\frac{1}{2}$ to 1 hour) may or may not damage the excretory function of the kidney.

EXPERIMENTAL UNILATERAL AND PARTIAL RENAL CORTICAL NECROSIS: ITS SIGNIFICANCE. Bernard Black-Schaffer* and Uriel Garcia-Caceres, University of Cincinnati, College of Medicine, Cincinnati, Ohio.

Occasional cases of unilateral renal cortical necrosis are encountered in man. The absence of the lesion in the one kidney may be understood if it is postulated that spasm of the renal arterial tree is the underlying cause of the lesion and that for some reason spasm was either absent in one kidney or that an extra-renal collateral circulation existed adequate to prevent significant injury despite severe spasm of the renal arteries.

Experimentally, both protective mechanisms may be demonstrated. On the one hand Schwartzman potent toxins directly inoculated into the kidney, presumably paralyzing its arterial tree, may be associated after provocation with cortical necrosis in the untreated kidney. On the other hand the operative production of an extra-renal collateral circulation may protect that portion of the kidney from the effects of staphylococcal toxin which produces cortical necrosis in the unmanipulated kidney as well as in the unprotected portion of the experimental organ.

MORPHOLOGIC CHANGES IN MALIGNANT NEPHROSCLEROSIS AFTER TREATMENT WITH POTENT ANTIHYPERTENSIVE DRUGS. Lawrence J. McCormack,* Jean Beland, and Roland E. Schneckloth, Cleveland Clinic, Cleveland, Ohio.

The extensive use of potent therapeutic agents in the management of malignant hypertension has led to striking changes in the renal findings at necropsy. This report is based on a study of the renal lesions found at necropsy in 12 patients with malignant hypertension treated with ganglion blocking agents and/or hydralazine, for 3 months to 3 years.

The criteria necessary for morphologic recognition of treated malignant nephrosclerosis will be presented and contrasted with the renal findings in 88 patients with malignant hypertension necropsied prior to treatment with these agents. At the time of death in these 12 patients, the necrosis and thrombonecrosis of small renal arteries and arterioles, characteristic of malignant nephrosclerosis, were found infrequently. Evidences of previous necrotizing renal lesions were suggested by the presence of organized thrombi, perivascular fibrosis, lamellar hyperplasia of small arterioles, and partial fibrosis of glomerular tufts. Atherogenesis of small vessels was no longer observed. Although the renal lesions were altered, uremia was still the most common cause of death in this select group of hospital patients.

SUBACUTE AND CHRONIC GLOMERULONEPHRITIS: HISTOPATHOLOGIC STUDY BY MEANS OF THIN SECTIONS.† Jacob Churg* and Edith Grishman, Mount Sinai Hospital, New York, N.Y., and Barnert Memorial Hospital, Paterson, N.J.

Twenty cases of subacute glomerulonephritis and 20 cases of chronic glomerulonephritis were studied by means of thin (0.5μ) sections, stained with periodic acid-Schiff reagent (PAS) and trichrome (chromotrope-aniline blue, CAB). It had been demonstrated that the essential features of acute glomerulonephritis are

† Supported by Research Grant (A-918(c)Path.) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service.

exudation of fluid and leukocytes and proliferation of large mononuclear cells in the intercapillary space of the glomerulus, causing compression of the capillaries. When complete resolution of the acute inflammation does not occur, deposition of fibers in the intercapillary space signals the onset of the subacute stage. While the histopathologic picture of acute diffuse glomerulonephritis is the same in every instance, differing only in degree, the subacute and chronic stages present various combinations of intercapillary inflammation and fibrosis, deposition of hyalin, "membranous transformation" of the wall and proliferation of epithelial cells, so as to impart almost an individual pattern to each case.

The intercapillary changes consist of accumulation of large mononuclear cells and fibers in the enlarged lobular centers. The cells are probably of histiocytic or connective tissue origin. The fibers stain blue with CAB and intensely red with PAS. They are twisted and gnarled, in contrast to the smooth wavy fibers seen in arteriosclerotic glomeruli. Deposition of hyalin is a frequent feature of subacute glomerulonephritis. This hyalin stains red with CAB and red with PAS. It is found not only in the fibrosed lobular centers, but also in the capillary walls, capillary lumina, and walls of arterioles.

Two forms of "membranous transformation" of the capillary wall are encountered. In one, there is deposition of hyalin on the inside of the capillary basement membrane. Such change is focal rather than diffuse, and may closely resemble the "wire loops" of disseminated lupus erythematosus. The hyalin is uniform or slightly vacuolated and is often separated from the capillary lumen by a second, very thin membrane. The other type of membranous transformation ("membranous glomerulonephritis" of Bell) is caused by formation of a broad zone on the outside of the basement membrane, between it and the epithelial cells. This zone consists of bands perpendicular to the basement membrane, alternately PAS positive and negative, or, respectively, blue and red with CAB. The periodicity is of the order of 0.5 to 1.0 μ .

The essential feature of glomerular obsolescence in chronic glomerulonephritis is collapse of the capillaries. This obsolescence can be brought about by fibrosis alone, by fibrosis and hyalinization, or by pure capillary collapse. In the latter case a seemingly hyalinized glomerulus consists of nothing but a tangle of basement membranes. These may be altered by the disease process, split, wrinkled, or the seat of membranous transformation; on occasion, they appear entirely normal.

DIETARY PRODUCTION OF LIPOGRANULOMA IN RATS. Alvin J. Cox, Jr.* and Floyd DeEds, Stanford University School of Medicine, San Francisco, Calif., and Western Utilization Research Branch, U.S. Department of Agriculture, Albany, Calif.

Within 60 days after the initiation of diets containing 10 per cent or more of acetostearin, the subcutaneous and abdominal fatty tissue of rats exhibits focal lesions resembling foreign body reactions around fat cells, which sometimes contain crystalline material. Changes in adipose tissue have not appeared after feeding of other types of fat. The lesions resemble those in human infants with sclerema adiposum neonatorum. A possible relationship to human lipogranuloma and to other forms of non-suppurative panniculitis is suggested.

STIMULATION OF ENTEROCHROMAFFIN CELL SECRETION BY RESERPINE AND FORMATION OF ACUTE GASTRIC AND DUODENAL ULCER IN ANIMALS.† Ruth L. Wong and Earl P. Benditt,* University of Chicago Clinics, Chicago, Ill.

Reserpine (5 mg. per kg. of body weight intraperitoneally) causes release of

† Supported in part by grants-in-aid from the Chicago Heart Association, the United States Public Health Service (Grant H-2141), and the Illinois Department of Welfare.

intestinal 5-hydroxytryptamine (serotonin) and the demonstrable enterochromaffin cell substance of guinea pigs. Associated with this there is necrosis of the epithelium of the villous tips of the duodenum and acute duodenal ulcers. The acute ulceration is evident during the first 24 hours after the drug is administered. Its maximal occurrence is from the 4th to the 16th hour. Healing is evident by the second day and little, if any, evidence of the lesions is seen after 5 days. The ulcers exhibit acute necrosis and include in some instances all the mucosa to the muscularis mucosae. They resemble acute peptic ulcers of man. Healing consists of re-epithelization of the ulcer crater.

In rats, hemorrhages in the mucosa of the glandular stomach are seen following reserpine treatment. The time of appearance is similar to that of the lesions of guinea pigs. Microscopic hemorrhage with minimal evidence of necrosis is seen in the lesions of rats. The rabbit shows hemorrhagic and ulcerative lesions in the stomach after reserpine. In the guinea pig and rabbit the lesions occur in the sites of largest numbers of enterochromaffin cells; in the rat the stomach has the least number of these cells. This suggests that some factor(s) other than or in addition to enterochromaffin cells and their secretion are important in the production of the ulcerative and hemorrhagic lesions. The secretion of acid gastric juice, which is known to be enhanced by parenteral reserpine, is one obvious factor. Others remain to be uncovered. These lesions present an interesting pathologic process resembling peptic ulcers and may be useful in the experimental elucidation of the pathogenesis of peptic ulcers.

EFFECT OF L-NOREPINEPHRINE ON THE PATHOLOGIC CHANGES OCCURRING IN HEMORRHAGIC SHOCK. Donald B. Hackel* and Bernard Catchpole, Western Reserve University School of Medicine at City Hospital, Cleveland, Ohio.

The present study attempts to clarify the nature of the cardiac and intestinal lesions that occur in hemorrhagic shock by analyzing the effects of l-norepinephrine therapy. Sixty dogs were heparinized and anesthetized (morphine, nembutal, and dial-urethane) in pairs, and were bled into a reservoir that was set at a height to maintain mean arterial pressure at 30 mm. of Hg. Both animals were kept oligemic for the same length of time (60 or 90 minutes), but one dog of each pair was given an infusion of l-norepinephrine at a rate that maintained his blood pressure at the pre-hemorrhagic level. At the end of the experiment the blood was returned. The dogs that survived were sacrificed after 4 to 7 days. There was no difference in the survival rates of the two groups. Hemorrhagic and necrotic lesions in the subendocardial region of the heart were present in a significantly greater number of dogs treated with l-norepinephrine. On the other hand, the incidence of hemorrhagic lesions in the intestinal mucosa was not significantly changed by l-norepinephrine therapy.

Previous metabolic studies in this laboratory have demonstrated a block in energy production in the hearts of dogs in shock. It is postulated that the subendocardial lesions are largely due to histotoxic anoxia. The administration of l-norepinephrine increases this tendency to anoxia by increasing the work of the heart. In contrast, the intestinal lesions are due presumably to passive congestion of the intestinal capillaries and venules, which is partly relieved by l-norepinephrine.

SHOCK FROM HOMOLOGOUS LYMPHOID TISSUE: ITS RESEMBLANCE TO TRYPSIN SHOCK. Herbert C. Stoerk,* Ritsu N. Arison, and Tatianja V. Budzilovich, Merck Institute for Therapeutic Research, Rahway, N.J.

Recently we have reported that a state of shock results in a variety of animal species when extracts of lymphoid tissue from a homologous source (or in some instances from a very closely related heterologous source) are injected intravenously. This state of shock was associated with mydriasis, dyspnea, prolonged

inactivity, and a striking delay of blood clotting. Larger doses of the extracts produced convulsions and death. Comparable extracts from lymphoid tissue of species foreign to the ones injected proved innocuous. Adrenalectomy or vaccination with pertussis did not increase the animal's susceptibility to this type of "shock." Antihistamines, atropine, adrenalin or pretreatment with cortisone failed to modify the animal's reaction to the extracts. However, small amounts of heparin completely prevented shock from otherwise fatal doses. Also, pretreatment with sublethal amounts of extract caused "desensitization" inasmuch as otherwise fatal doses were well tolerated by such animals. A certain resemblance became apparent between shock resulting from homologous lymphoid tissue and that produced by intravenous injection of large amounts of trypsin. Trypsin-shock rather closely duplicated the symptoms of fatal shock from homologous lymphoid tissue and was also prevented by heparin or by pretreatment with sublethal doses of trypsin. That shock from homologous lymphoid tissue (HLT) was not due to a trypsin-like action of the extracts became evident from the fact that fatal doses of extracts of HLT were completely devoid of proteolytic activity for protein of the same or that of another species. Also, while after the injection of HLT, shock resulted only after a delay, injections of trypsin are instantaneously followed by severe symptoms.

SELECTIVE RADIATION INJURY TO CARTILAGE WITH RADIOACTIVE SULFUR.
R. L. Swarm, P. Rubin, and K. Brace, National Cancer Institute, Bethesda, Md.

S^{35} given in the form of the inorganic sulfate administered intraperitoneally is deposited in cartilaginous tissue. The biologic half-life of radioactive sulfur deposited in cartilage is greater than the biologic half-life of radioactive sulfur deposited in other tissues. When large amounts of S^{35} were given to young rats, the radiation from the radioactive sulfur caused cessation of cartilage growth and histologic alterations in cartilage which were similar to the histologic alterations produced in cartilage by intense local x-irradiation. The alterations found in cartilage included enlarged atypical chondrocytes, disruption of the columnar arrangement of the chondrocytes, irregularity in the staining of the intercellular matrix, and a decrease in the number of chondrocytes. Coincident with these changes in cartilage there was cessation of growth manifested by failure of the long bones to increase in length. Alteration of cartilage was most extensive in the sites of greatest growth such as the epiphyseal cartilage plates of the long bones and the costochondral junctions of the ribs. Radiation changes were proportional to the amount of S^{35} incorporated in cartilage. The occurrence of greater alteration of the cartilage in sites of greater growth is partially explained by the fact that the incorporation of S^{35} into cartilage is related to the rate of growth of the cartilage. The incorporation of S^{35} into cartilage is increased in younger animals with faster growth rates. No extensive alteration of the bone and soft tissue adjacent to the cartilage was found. Some information regarding the extent of damage to tissues other than cartilage following the administration of large amounts of S^{35} will be presented. The distribution of sulfur to other parts of the body produced some changes similar to whole body x-irradiation.

NEOPLASMS IN MONKEYS (*MACACA MULATTA*): SPONTANEOUS AND IRRADIATION INDUCED. Sidney P. Kent and John E. Pickering, Radiobiological Laboratory of the University of Texas and the U.S. Air Force, Austin, Texas; and School of Aviation Medicine, U.S. Air Force, Randolph Air Force Base, Texas.

Spontaneous neoplasms are said to be rare in monkeys. Further, attempts to produce malignant neoplasms in monkeys using compounds that are known to be carcinogenic in other animals have, for the most part, been unsuccessful. Presumably there is a relationship between the apparent low instance of spontaneous

neoplasms in monkeys and the difficulty in experimentally producing neoplasms in this species.

A colony of monkeys (*Macaca mulatta*) has been maintained at the Radiobiological Laboratory, Balcones Research Center, of the University of Texas and the United States Air Force, Austin, Texas, for the past 6 years. During this period material has been collected from over 450 necropsies. At the present time, there are 417 living animals in the colony. Nine neoplasms have been found in this material: fibroma of the adrenal gland, papillomas of the stomach, pituitary adenoma, hemangio-endothelioma of the subcutaneous tissue, osteochondrosarcoma, malignant lymphoma, two fibrosarcomas, and a glioblastoma multiforme. The glioblastoma and the two fibrosarcomas occurred in animals receiving focal ionizing irradiation under circumstances suggesting that they were induced by the irradiation. The other six neoplasms occurred in animals that had not been exposed to a known carcinogen, or had been exposed under circumstances suggesting that the carcinogen was not related to the development of the neoplasm. The malignant lymphoma and the glioblastoma are of further interest in that they are the first reported cases of these types of neoplasms in monkeys.

TEN-YEAR FOLLOW-UP OF 266 CASES OF TESTICULAR TUMORS. R. M. Mulligan,* University of Colorado School of Medicine, Denver, Colo.

Two hundred and sixty-six cases of testicular tumors from the Armed Forces Institute of Pathology were divided into four types on the basis of the most malignant neoplastic tissue in each tumor as follows: trophoblastic (chorionepithelioma)—13, immature somatic (malignant teratoma, teratocarcinoma, embryonal carcinoma)—147, seminal (seminoma)—95, and mature somatic (benign or adult teratoma)—11. Follow-up information was obtained in 252 cases (94.7 per cent) by the Follow-Up Unit of the Armed Forces Institute of Pathology. In 239 cases, the results were clear-cut, with 126 patients dead and 113 alive and free of known disease 10 years or more after orchiectomy. The mortality figures in these 239 cases were as follows: trophoblastic, 11 of 12 dead or 91.7 per cent; immature somatic, 99 of 138 dead or 71.7 per cent; seminal, 15 to 81 dead or 18.5 per cent; and mature somatic, 1 of 8 dead or 12.5 per cent. The remaining 13 cases included 1 immature somatic free of known disease, 7¾ yrs.; 1 seminal surviving 7½ yrs.; 1 seminal alive 7 yrs.; 1 immature somatic operated on for metastatic disease 4 yrs. after orchiectomy alive 12 yrs.; 1 immature somatic operated on for metastatic disease 12 yrs. after orchiectomy; 1 case with immature somatic tumor and 2 with seminal neoplasms developing seminoma of the opposite testis 9½ yrs., 4½ yrs., and 12 5/6 yrs. after original orchiectomy; 1 patient with immature somatic tumor dying of duct carcinoma of the pancreas 12 yrs. after orchiectomy; 1 patient with seminal tumor dying of coronary arterial thrombosis 12 1/6 yrs. after orchiectomy; another dying of coronary arterial insufficiency 11½ yrs. after orchiectomy; 1 patient with mature somatic tumor dying of skull fracture 10 11/12 yrs. after orchiectomy; and 1 patient with mature somatic lesion dying of arteriosclerosis 11¾ yrs. after orchiectomy.

READ BY TITLE

ORGANIZATION BY SMOOTH MUSCLE CELLS. M. Daria Haust, Henry Z. Movat, and Robert H. More,* Queen's University Faculty of Medicine, Kingston, Ont.

In the course of morphologic and histochemical studies on various lesions of arteriosclerosis, we have noticed an avascular form of organization of blood proteins by cells which possessed all the morphologic attributes of smooth muscle cells.

The nucleus of these elongated, slender cells was long, wavy, and cigar-like in shape with rounded, blunt ends. It was situated in the widest portion of the cell body, and was placed slightly off the center on a transverse cut. In a longitudinal section the nucleus exhibited a wrinkled, snake-fence appearance, its length and shape being the expression of the degree of contraction of the cell. Its granular chromatin was uniformly distributed; it possessed usually one, at times two, nucleoli.

The cytoplasm of these cells was elongated, slender, and tapering. It was pale eosinophilic and within its most homogeneous matter one could distinguish numerous fine, strongly eosinophilic myofibrils and at the periphery coarse, similarly stained myofibrils, running parallel to the longitudinal axis of the cell. They were PTAH-positive and stained a deep red with HPS, Masson's trichrome, and Heidenhain's azan. The myofibrils of the individual cells were interlacing with the neighboring ones, forming a net-like arrangement.

Each of these cells was surrounded by a fine reticulum of PAS-positive fibers and by elastic fibers, forming a coat-like covering. This latter feature is also known to be a characteristic of the smooth muscle cells of the cardiovascular system.

None of these above described features were demonstrable on known fibroblasts.

In ageing, these cells showed the morphologic features of old smooth muscle cells.

The origin of these cells is uncertain. Since they normally arise from mesenchyme, it would appear probable, in accordance with Marchand, that they may originate from the undifferentiated mesenchymal, perivascular (pericapillary) cells. On the other hand, as suggested by Benninghoff, "They might arise from fibroblasts," since he found all forms of intermediary stages from fibroblasts to smooth muscle cells. We, too, have found closely related forms of both cell types. Finally, some authors (Crawford and Levine) believe that these cells (which, however, they have not named smooth muscle cells) originate either from blood (monocytes) or from intimal epithelium.

THE PATHOGENETIC SIGNIFICANCE OF THE VARIOUS CELLS IN THE ARTHUS, PASSIVE ARTHUS, AND LOCAL PRIMARY IMMUNE RESPONSE. Henry Z. Movat and Robert H. More,* Queen's University Faculty of Medicine, Kingston, Ont.

The morphologic specificity of the tissue reactions at sites of local immune response remains controversial. In the course of studying the morphologic responses at serial time intervals, in the Arthus, the passive Arthus, and the primary immune response to injected antigen, certain components and patterns of reaction have occurred which appear to show highly characteristic although not specific morphologic features for the type of immune phenomena occurring in the tissues.

In both the Arthus and passive Arthus reaction there was, within the first 24 hours after injection of the antigen, an acute serofibrinous inflammation with infiltration of some polymorphonuclear leukocytes. In both of these phenomena, the local immunologic state is set for an antigen-antibody reaction. In contrast, no acute exudative lesion occurred following the primary injection of alum precipitated horse serum.

A proliferation of macrophages was common to the Arthus, passive Arthus, and site of a single injection of alum precipitated horse serum within 24 hours of the injection. It was best seen in the alum precipitated horse serum injections, when the cytoplasm of the macrophages contained vacuoles staining similarly to the intercellular antigen.

Formation of plasma cells also was common to each of the three different local immunologic reactions but they varied in their time of appearance. In the Arthus reaction pro-plasmacytes were present in the first 12 to 24 hours and were mature in 4 to 5 days. On the other hand pro-plasmacytes did not appear until

the 4th or 5th day in the passive Arthus or following the injection of the alum precipitated horse serum, and did not mature until the 8th to 14th day.

The type of cell response and sequence of reaction in these three different local immunologic phenomena may be explained on the following basis. The acute serofibrinous inflammation of the Arthus and passive Arthus represents the tissue response to a local antigen-antibody reaction. This immunologic phenomenon only occurs in these two experimental conditions of those described. The macrophages, on the other hand, represent the response to the antigen followed by ingestion. The immunologic state would allow this phenomenon to occur immediately in all three experimental situations described. Finally, the plasma cells are the morphologic counterpart of antibody formation, occurring in 4 days as a secondary immune response in the previously sensitized animals with the Arthus reaction and occurring in 8 to 14 days as a primary immune response in the previously unsensitized animals of the passive Arthus and primary antigen injected groups.

CORRELATION OF HISTOLOGIC, SEROLOGIC, AND ELECTROPHORETIC DATA IN SUBCLINICAL RHEUMATIC CARDITIS. K. C. Pani and Bernard M. Wagner,* Children's Hospital of Philadelphia, Philadelphia, Pa.

With the advent of surgical methods for the correction of rheumatic cardiac valvular disease, heart tissue has become available for microscopic examination. Prior to surgery patients are thoroughly evaluated clinically including fluoroscopy, x-ray, electrocardiogram, and cardiac catheterization when indicated. Laboratory studies include complete blood count, sedimentation rate, C-reactive protein, anti-streptolysin, antifibrinolysin, and paper electrophoresis. Surgery is carried out only when the clinical and laboratory data indicate inactive rheumatic heart disease. During surgery, the right or left auricular appendage is biopsied. In the postoperative period, the laboratory studies are repeated. A total of 40 patients has been evaluated in this manner. The relationship of the tissue findings to the laboratory data will be presented. Special attention will be directed to those cases demonstrating the presence of Aschoff bodies in the biopsy specimens. In keeping with other information, it appears that the Aschoff body as the pathognomonic feature of rheumatic carditis does not correlate with the clinical and laboratory findings.

DETERMINATIONS OF GLUCOSE AND NON-PROTEIN NITROGEN IN BLOOD AND SPINAL FLUID AT NECROPSIES OF MENTALLY SICK PATIENTS DURING DIFFERENT TIME INTERVALS AFTER DEATH AND IN VARYING FATAL DISEASES, AND THEIR DIAGNOSTIC EVALUATION. George Strassmann,* Metropolitan State Hospital, Waltham, Mass.

Determinations of glucose and non-protein nitrogen content of blood and spinal fluid were made on material obtained at the necropsies of mentally sick patients, most of them over 60 years of age and dying from varying diseases including diabetes. The values obtained during time intervals ranging from 1 to 49 hours after death were compared. In some instances results of similar examinations during life shortly before death were compared also with the postmortem determinations.

Tabulations of the results show a high but very variable glucose curve of the blood of the right heart and generally low amounts in the spinal fluid. The results in diabetes varied according to the insulin treatment or insulin resistance before death. Hyperglycemia was a rule in most necropsies performed during the first 10 hours after death, but exceptions occurred. Neither age nor nutrition nor fatal disease seemed to play a significant rôle in this respect. This hyperglycemia is an unspecific stress reaction resulting in adrenal stimulation and mobilization of glucose from sugar depots in the body. Not enough blood was obtained in

most cases from the left heart for an exact determination of the glucose. Some glucose disappears frequently from the blood in late necropsies performed 15 hours or more after death, similarly as the glucose content of the blood and spinal fluid decreases if kept *in vitro* at room temperature. Very high glucose amounts (400 mg. per cent and more) were observed in necropsies done after 26 and more hours in instances of insulin-resistant diabetes.

Hyperglycemia in early necropsies after death is an unspecific reaction, and has therefore no value for the diagnosis of diabetes. Spinal fluid contains at post-mortem less amounts of glucose than normal in the majority of necropsies performed at any time. The mobilized glucose apparently does not pass through the blood-spinal fluid barrier. High glucose values in the spinal fluid are observed in cases of diabetes and have therefore more diagnostic importance than a hyperglycemia.

In contrast to the glucose, the non-protein nitrogen of blood and spinal fluid is usually high and remains constantly high at all times. It also does not decrease *in vitro* from these fluids. It may indicate only an unspecific failure of the kidneys to excrete these substances from the blood. Signs of a true uremia due to an advanced nephrosclerosis or pyelonephritis were present in a number of cases. High non-protein nitrogen values were not explained by such diseases in cases of so-called sudden death by coronary occlusions or pulmonary embolism or other instances and were considered as of an unspecific nature, a terminal event.

High glucose and non-protein nitrogen contents of blood and spinal fluid obtained from necropsies have only a limited value for the diagnosis of metabolic or kidney diseases preceding death. Such increase is observed frequently in a variety of fatal diseases, in non-diabetic patients and even in instances of sudden death.

